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Nodal Injection of *Agrobacterium tumefaciens* for Gene Functional Analysis in Peanut: An Appraisal

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Abstract: Peanut is a key cash crop worldwide, yet the limited availability of functional genes and markers for breeding hinders further progress, largely due to the lack of an efficient and user-friendly transformation system. This study aimed to comprehensively evaluate the effectiveness of nodal agroinjection, a novel transformation technique we developed for peanut, by introducing the soybean cold-tolerance gene *SCTF-1*. Putative transgenic seeds and seedlings were screened using genomic DNA PCR, while transgene expression was analyzed via qRT-PCR and phenotypic assessments. Southern blotting confirmed the stable integration of *SCTF-1*. The transgenic seedlings displayed enhanced chilling tolerance, characterized by increased proline accumulation, reduced malondialdehyde (MDA), and elevated peroxidase (POD) activity. These findings demonstrate that nodal agroinjection is an efficient and reliable approach for generating transgenic peanut and analyzing gene function. This method offers a promising alternative to conventional tissue culture-based transformation strategies.

Keywords: *Agrobacterium tumefaciens*; peanut; genetic transformation; cold tolerance; nodal injection

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1. Introduction

Peanut (*Arachis hypogaea* L.) is a versatile cash crop that is valued as a source of oil, food, vegetables, fodder, tea, and medicine [1–4]. It holds significant importance in agricultural industries and rural economies. Achieving breakthroughs in peanut breeding hinges on identifying and utilizing novel genes. However, the pool of genes and molecular markers available for peanut genetic improvement is notably constrained. Although progress in sequencing technologies has facilitated differential gene expression analysis and quantitative trait locus (QTL) mapping using hybrid and natural populations, the functional validation of candidate genes remains indispensable. Unfortunately, the limitations of current peanut genetic transformation systems have become a critical bottleneck, hindering the translation of these discoveries into practical breeding outcomes.

Traditional peanut transformation methods rely heavily on tissue culture, which poses significant challenges due to strong genotype dependency. Genotypes with high regeneration capacities are not necessarily amenable to transformation; conversely, easily transformable genotypes may exhibit poor regeneration potential, and even genotypes that are both transformable and regenerable may lack agricultural or economic relevance.

To address the challenges, transformation techniques that bypass tissue culture have been explored. Among these, the pollen tube pathway method involves injecting *Agrobacterium tumefaciens* into open flowers [5,6], while needle-pricking randomly creates wounds on the embryo axes of mature seeds with a single cotyledon (incubated on semisolid Murashige and Skoog (MS) basal medium [7] for two days prior to infection), followed by immersion in an *A. tumefaciens* suspension to achieve transformation [8]. There have been some reports on the application of both techniques, but their use remains rather limited. To date, most studies on transgenic peanut primarily use genomic DNA PCR, RT-PCR, and qRT-PCR to confirm the presence of transgenes, with only a limited number incorporating Southern blot analysis to verify stable integration.

The nodal injection method, also termed node injection [9,10] or nodal agroinjection [11], developed by our team, represents a promising alternative for peanut transformation. This technique is designed to leverage the reproductive biology of peanut plants, targeting the first pair of lateral branches, which contribute 60–70% of the total yield. Specifically, the method involves injecting *A. tumefaciens* carrying the desired gene construct into the first and second nodes of cotyledonary branches. This allows for the transformation of germline cells associated with seed formation.

This approach offers several advantages. With multiple flowers typically present at each node, a single injection can yield multiple pods, enhancing efficiency and lowering seed requirements compared to the pollen tube pathway and needle-pricking methods. Additionally, this technique circumvents the need for tissue culture, reducing genotype dependency. Moreover, it has already been successfully applied to produce CRISPRed high-oleic peanut [10]. In a separate study, transgene expression was confirmed through ELISA (enzyme-linked immunosorbent assay) and peanut DNA sequences flanking the T-DNA insertion sites were identified, providing evidence for the integration of foreign genes into the peanut genome [12]. However, certain limitations persist, particularly the lack of Southern blot analysis to verify stable transgene integration—a step that is widely regarded as essential for validating the reliability of this method.

In high latitude, cooler regions, the risk of aflatoxin contamination in peanut is low. However, peanut cultivation in these areas is constrained by a short frost-free growing season, with low temperatures during sowing, the seedling stage, or late growth stages often leading to significant yield losses [13]. While seed coatings that enhance peanut seed resistance to both low temperatures and high humidity can mitigate cold stress during sowing [14], and early-maturing varieties can address late season cold stress, recent studies have shown that foliar application of calcium (Ca^{2+}) can alleviate chilling stress at the seedling stage by improving photosynthetic capacity [15]. However, effective agronomic practices to fully counter cold stress at the seedling stage in commercial production remain insufficient.

The *SCTF-1* gene identified in soybean and, like peanut, a member of the Fabaceae family and the Papilionoideae subfamily, has been demonstrated to confer cold tolerance [16]. However, its introduction into peanut and its functional effects in this crop remain unexplored. Nevertheless, it offers a promising avenue for mitigating the susceptibility of peanut to low temperature stress.

One aim of this study was to address this gap by introducing *SCTF-1* into peanut using nodal injection transformation. The primary objective of this research was to evaluate the effectiveness of nodal injection transformation for gene functional analysis and transgenic plant production, utilizing multiple techniques—including gene expression analysis, Southern blotting, and biochemical measurements—to establish a foundation for its broader application.

2. Materials and Methods

2.1. Peanut Materials

Six peanut landraces from Jilin Province, China, ZH1071, ZH1105, ZH1160, ZH1059, ZH1085, and ZH1069, were selected for this study based on a prior evaluation of chilling tolerance during the germination and seedling stages (Table 1). Among these, ZH1071 and ZH1105 were classified as tolerant at the germination stage, exhibiting 100% and 76.91% germination, respectively, under low temperature conditions. In contrast, ZH1160, ZH1059, ZH1085, and ZH1069 were considered sensitive, with germination rates of only 23.82%, 33.33%, 4.76%, and 4.76%, respectively [17]. At the seedling stage, ZH1071 demonstrated high tolerance, while the remaining landraces were categorized as highly sensitive or sensitive [17].

Table 1. Information about peanut landraces used in this study [17].

Peanut Landrace	Days to Maturity	Response to Chilling Stress at Germination Stage	Response to Chilling Stress at Seedling Stage
ZH1160	120	Sensitive	Highly sensitive
ZH1105	120	Tolerant	Sensitive
ZH1059	120	Sensitive	Highly sensitive
ZH1085	120	Sensitive	Sensitive
ZH1071	120	Tolerant	Highly tolerant
ZH1069	120	Sensitive	Sensitive

2.2. Transformation of A. tumefaciens

An *Escherichia coli* strain carrying the expression vector pCPB-SCTF-1, which contains the soybean *SCTF-1* gene under the control of the 35S promoter, previously constructed in Prof. Peiwu Wang's laboratory [16] (Figure 1), was used in this study. Using a sterilized loop, stored *E. coli* cells were transferred onto a petri dish containing solid LB growth medium supplemented with 100 mg/mL kanamycin [18]. The plates were incubated overnight at 37 °C. The next day, single colonies were picked and inoculated into test tubes containing 5 mL liquid LB medium supplemented with 5 µL of 100 mg/mL kanamycin. The cultures were incubated overnight at 37 °C with shaking at 200 rpm. Plasmid DNA was extracted from the *E. coli* cultures using a Plasmid Minspin HP Kit (Vigorous Biotechnology, Beijing China) according to the manufacturer's instructions. The plasmid DNA was stored at −20 °C for subsequent use.

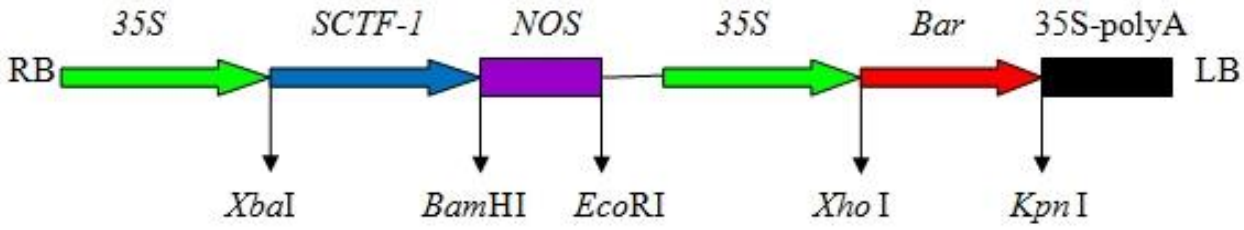

Figure 1. Partial map of plant expression vector pCPB-SCTF-1, showing the region between right and left borders [17].

The expression vector pCPB-SCTF-1 was introduced into competent cells of *Agrobacterium tumefaciens* strain EHA105 following the protocol of Wise et al. [19], with slight modifications. Briefly, 7.5 µL of plasmid DNA (0.5 µg/µL) was added to frozen competent cells prior to thawing. The cell–DNA mixture was frozen in liquid nitrogen for 1 min and then thawed in a 37 °C water bath for 5 min. Subsequently, 1 mL of Yeast Extract Peptone (YEP) medium (without antibiotics) was added to the mixture in a 1.5 mL Eppendorf tube.

The suspension was incubated at 28 °C with shaking at 140 rpm for 3 h. After incubation, cells were collected by centrifugation at 6000 rpm for 5 min, resuspended in 1 µL of YEP medium, and plated on solid YEP medium containing 100 mg/mL kanamycin to isolate single colonies.

Single colonies of *A. tumefaciens* EHA105 carrying the expression vector pCPB-SCTF-1 were selected and cultured overnight in 5 mL liquid YEP medium containing 5 µL of 100 mg/mL kanamycin at 28 °C with shaking at 200 rpm. PCR was performed using the SCTF-1-F and SCTF-1-R primers (Table 2) [16] to verify the positive colonies. The 25 µL PCR mixture consisted of 12.5 µL 2× Taq Platinum Master Mix (Tiangen Biotech, Beijing, China), 1 µL of each primer, and 2 µL of YEP containing the putative positive *Agrobacterium* cells. The PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 54.5 °C for 40 s, and 72 °C for 40 s, with a final extension at 72 °C for 5 min. PCR products were separated on a 1% agarose gel and visualized using Gelview stain (BioTeke, Wuxi, China).

Table 2. Information about primers used in this study [16].

Primer Name	Primer Sequence (5′-3′)	Length of PCR Product (bp)	Use
SCTF-1F, SCTF-1R	Atggctttggaagctcttca, gtattgagggatttcaatcttgggt	699	Colony PCR, seed PCR, seedling PCR, RT-PCR, and probe preparation
BarF, BarR	tcaaatctcggtgacgggc, atgagcccagaacgacgc	552	Seedling PCR
NOSF, NOSR	gaatcctgttgccggtcttg, ttatcctagtttgcgcgcta	250	Seedling PCR
QSCTF-1F, QSCTF-1R	aggttcttcgttgatgac, atttcaatcttgggtatggt		qRT-PCR
QTUA5F, QTUA5R	gctcggcttcaccatctac, aagagcacagcgacatcag		qRT-PCR (reference gene)

2.3. Nodal Injection of *A. tumefaciens*

A 500 µL aliquot of PCR-verified *A. tumefaciens* suspension was cultured in 200 mL liquid YEP medium supplemented with 200 µL of 100 mg/mL kanamycin in a 500 mL flask. The culture was incubated at 28 °C with shaking at 200 rpm until the optical density at 600 nm (OD_{600}) reached 0.7, as measured using a Shimadzu UVmini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The cells were then collected by centrifugation at 7000 rpm for 5 min and resuspended in WinansAB* liquid medium, prepared according to the technique described by Wise et al. [19]. The *Agrobacterium* suspension was incubated at 28 °C with agitation at 150 rpm for 2 h prior to injection.

Thirty peanut plants were cultivated for each landrace. For nodal injection, the freshly prepared bacterial suspension was introduced into the first and second nodes of cotyledonary branches of peanut plants using a 4 mL disposable syringe, 30 d after sowing (DAS) and before flowering, following the protocol of Wang et al. [9].

2.4. Molecular Analysis of Putative Transgenic Seeds

Transgenic peanut seeds (T_0) were identified by PCR amplification of the *SCTF-1* gene, using genomic DNA extracted from each seed. To minimize adverse effects of sampling on seed germinability, 3–5 mg of cotyledonary slices distal to the embryo end were collected. DNA extraction was performed using a CWBIO kit (CWBio, Beijing, China) following the manufacturer's instructions.

The 25 µL PCR reaction mixture contained 12.5 µL 2× Taq Platinum Master Mix (Tiangen Biotech, Beijing, China), 8.5 µL sterile double-distilled water, 1 µL of each *SCTF-*

1-specific primer (SCTF-1F/SCTF-1R, Table 2) at 10 μmol/L, and 2 μL of DNA template. The PCR program followed the same conditions as those used for verifying *A. tumefaciens* transformant colonies and described in Section 2.2.

2.5. Molecular Analysis of Transgenic Plants Through Genomic DNA PCR

Genomic DNA was extracted from leaflet tissues (3–5 mg per sample) of seedlings derived from *SCTF-1* PCR-positive seeds and wild-type seeds. To reconfirm the transgenic seedlings, PCR was conducted using primers NOSF/NOSR and BarF/BarR, targeting the *NOS* and *Bar* genes, respectively, alongside SCTF-1F/SCTF-1R primers (Table 2, Figure 1).

The PCR conditions for amplifying the *Bar* and *NOS* genes were nearly identical to those used for detecting transgenes in seeds, except for the annealing temperatures, which were set at 50 °C for 30 s for the *NOS* primers and 58 °C for 30 s for the *Bar* primers. The PCR mixture composition was the same as that used for detecting the *SCTF-1* gene in seeds.

2.6. Plant Growth, Chilling Treatment, Sampling and Phenotyping for Chilling Tolerance

PCR-positive peanut seeds were planted in nutrient-rich soil within a growth chamber maintained under a 12-h photoperiod, with temperatures set to 28 °C during the light phase and 22 °C during darkness. Before being fully covered with soil, the seeds were treated with a 0.05% (*w*/*v*) Ethrel solution combined with 0.625 mL/L of a tebuconazole–carbendazim fungicide mixture. Fifteen days after emergence, samples were collected from various plant organs, flash-frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis.

In a pilot study, chilling stress treatments at 2 °C, 5 °C, and 10 °C were tested. Results indicated that 10 °C did not induce phenotypic differences among peanut genotypes within a short period of time, while 2 °C was lethal within 48 h. Based on these observations, 5 °C was selected as the optimal chilling stress condition.

The growth chamber temperature was subsequently lowered to 5 °C for 95 h, with consistent moisture levels maintained to prevent drought-related effects. Following the chilling treatment, samples were collected and stored at −80 °C. The plants were then returned to room temperature (~25 °C) for 5 d to assess recovery.

Plants that fully recovered within 2 d without leaf loss were classified as highly tolerant. Genotypes that shed basal leaves between days 2 and 5 were categorized as sensitive, while those that lost both basal and cauline leaves were classified as highly sensitive.

2.7. Molecular Analysis of Putative Transgenic Plants Through Reverse Transcription PCR (RT-PCR), and qRT-PCR

Total RNA was extracted from leaves, stems, and roots and sampled both immediately before and after the chilling treatment using the Trizol method. Complementary DNA (cDNA) was synthesized from the total RNA using an All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia™, Rockville, MD, USA) according to the manufacturer's protocol.

For RT-PCR, a 25 μL reaction mixture was prepared, comprising 13 μL PCR master mix, 8 μL sterile double-distilled water, 2 μL first-strand cDNA template, and 1 μL SCTF-1-specific primers (SCTF-1-F/SCTF-1-R) (10 μmol/L each). The PCR conditions were identical to those used for identifying positive seeds. PCR products were separated on an agarose gel stained with Gelview (BioTeke, Wuxi, China), and the expected bands were excised, purified, and assayed by Sanger sequencing (Sangon Biotech, Shanghai, China).

Quantitative real-time PCR (qRT-PCR) was conducted to quantify SCTF-1 gene expression in transgenic peanut plants, using the *TUA5* gene as a reference, following the recommendations of Chi et al. [20]. Each 20 μL qRT-PCR reaction mixture included 10 μL 2× All-in-One™ qPCR Mix (GeneCopoeia™, MD, USA), 3.9 μL sterile double-distilled water, 2 μL *SCTF-1*-specific primers (QSCTF-1F/QSCTF-1R), 2 μL reference gene-specific primers (QTUA5F/QTUA5R) (Table 2), 0.1 μL ROX reference dye, and 2 μL cDNA template.

The reactions were conducted using an Agilent Stratagene Mx3000P system (Agilent Technologies, Santa Clara, CA, USA) under the following two-step qRT-PCR conditions: initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 30 s. Threshold cycle (Ct) values were calculated using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [21]. Each landrace transformant-temperature combination had 1 biological replicate and 3 technical replicates.

2.8. Southern Blot Analysis of Genomic DNA PCR-Positive Plants

For the Southern blot analysis, genomic DNA from PCR-positive plants (one plant per recipient landrace) and untransformed control plants was digested overnight at 37 °C using the *Hind*III enzyme. The digested DNA was separated by electrophoresis on a 0.8% agarose gel without Gelview (BioTeke, Wuxi, China). Hybridization was performed using digoxigenin (DIG) (Roche Diagnostics, Basel, Switzerland)-labeled probes specific to the *SCTF-1* gene, prepared from PCR products of the pCPB-SCTF-1 plasmid DNA using the SCTF-1F/R primer pair (Table 2). Southern blot hybridization was performed using a DIG DNA Labeling and Detection Kit I (Roche, Basel, Switzerland), following the manufacturer's instructions.

2.9. Biochemical Assay

2.9.1. Proline Content

Proline content was determined using the sulphosalicylic acid–ninhydrin method, as described by Zhang and Huang [22]. Briefly, approximately 500 mg of leaf samples (in triplicate) were ground into powder with liquid nitrogen. The powdered tissue was homogenized in 2 mL of sulphosalicylic acid and centrifuged at 5000 rpm for 5 min. The supernatant was collected and 1 mL each of acetic acid and ninhydrin reagent was added. The mixture was incubated at 100 °C for 45 min, after which the reaction was terminated by cooling on ice for 30 min. An equal volume of toluene was added to the cooled mixture, which was then vigorously mixed and centrifuged at 1000 rpm for 5 min. Absorbance was measured at 520 nm, with toluene serving as the blank. A standard curve ($y = 0.0079x$, $R^2 = 0.9384$) was generated using serial proline standards (1–300 µM). The proline concentration was calculated in µmol/g fresh weight based on the method of Bates et al. [23]. The analysis for each sample was performed in three replicates (technical replicates). Each line had 2 biological replicates.

2.9.2. Malondialdehyde (MDA) Content

The malondialdehyde (MDA) content was estimated using the trichloroacetic acid (TCA)–thiobarbituric acid (TBA) method, according to Zhang and Huang [22], with minor modifications. The supernatant was mixed with 20% TCA and 0.5% TBA, followed by boiling at 100 °C for 15 min. After cooling, the absorbance was measured at 532 nm and 600 nm. The MDA content was calculated using the formula proposed by Heath and Packer [24]:

$$\text{MDA (nmol/mL)} = (OD_{532} - OD_{600})/155{,}000 \times 10^6.$$

Each sample was analyzed in triplicate. Each line had 2 biological replicates.

2.9.3. Peroxidase (POD) Activity

Approximately 500 mg of peanut leaves were homogenized with 100 mmol/L cold phosphate buffer (pH 6) using an ice-cold mortar and pestle. The homogenate was centrifuged at 4500 rpm for 13 min at 4 °C. The resulting supernatant was transferred to a fresh test tube placed on ice. The sediment was re-suspended in an equal volume of phosphate buffer, mixed thoroughly, and centrifuged under the same conditions. One

milliliter of the supernatant was mixed with 3 mL of reaction buffer, consisting of 50 mL phosphate buffer, 28 μL guaiacol, and 19 μL H_2O_2. Peroxidase (POD) activity was determined spectrophotometrically at 470 nm by measuring the change in absorbance due to guaiacol oxidation. Throughout the procedure, all samples were maintained on ice to prevent enzyme degradation. POD activity was calculated as described by Li [25] using the following formula:

$$\text{POD } (\text{U}\cdot\text{g}^{-1}\cdot\text{min}^{-1}) = =\Delta\text{OD}_{470} \times \text{VT}/\ (\text{W} \times \text{VS} \times 0.01 \times \text{t})$$

where ΔOD_{470} is the change in absorbance at 470 nm over 3 min, VT is the total volume of the extract (mL), VS is the volume of the enzyme solution used (mL), t is the reaction time (minutes), and W is the fresh weight of the sample (g) [26].

Each line had 2 biological replicates. Each sample had three replicates.

2.10. Statistical Analysis

Analysis of variance (ANOVA) was employed to evaluate differences in the measured parameters between transgenic seedlings and their respective controls in qRT-PCR and biochemical indicator results. Multiple comparisons were performed using the Tukey method in the analysis of qRT- PCR results. For biochemical indicator results, when significant variation was detected among individual means, a Student's t-test was conducted to further assess pairwise differences. All statistical analyses were performed using Statistical Analysis Software (SAS), version 9.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Identification of A. tumefaciens Transformants

The transformation of *A. tumefaciens* (EHA105) with the pCPB-SCTF-1 construct (Figure 1) was successful, as confirmed by the presence of a PCR-amplified band of the expected size (699 bp) (Figure 2). Single colonies of *A. tumefaciens* EHA105*A* transformants were used to prepare bacterial suspensions for nodal injection.

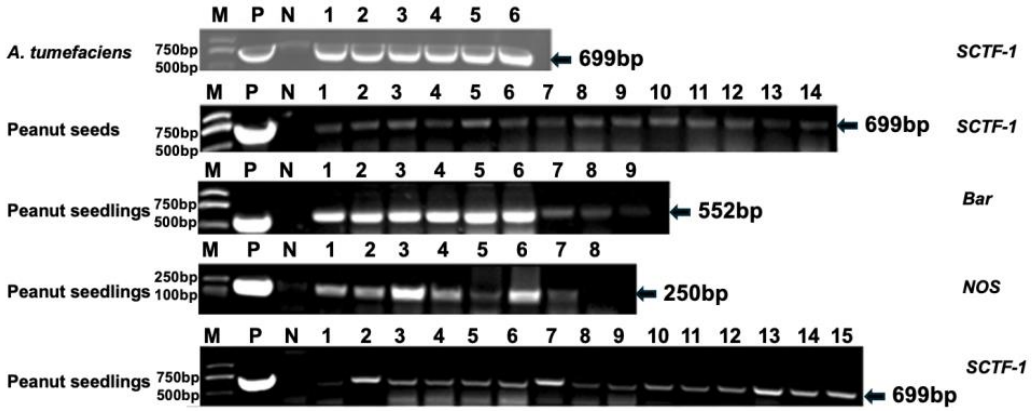

Figure 2. DNA PCR identification of *A. tumefaciens* transformants carrying the pCPB-SCTF-1 construct, along with putative transgenic seeds derived from 6 landraces and seedlings derived from 4 landraces, with the lengths of PCR-amplified products (bp) specified. M: 2000 bp DNA marker; P: positive control; N: untransformed negative control. The numbers above the lanes represented individual colonies, seeds, and seedlings. A single primer pair (SCTF-1F/R) was used for *A. tumefaciens* and seeds, whereas three primer pairs (BarF/BarR, NOSF/NOSR, and SCTF-1F/R) were used for seedlings.

3.2. Genomic DNA PCR Screening of Peanut Seeds and Seedlings Resulting from Nodal Injection

This study investigated the effectiveness of nodal injection of *A. tumefaciens* in six peanut landraces. A total of 2734 peanut pods were harvested, which, after hand shelling, produced 3115 peanut seeds. (Table 3). Putative transgenic peanut seeds were identified by genomic DNA PCR using *SCTF-1* primers and DNA was extracted from slices of cotyledons of 625 randomly sampled T_0 seeds (Figure 2).

Table 3. Overview of the number of peanut pods and seeds harvested following nodal injection of *A. tumefaciens*, the number of samples screened by genomic DNA PCR, and the number of positives.

Recipient	Pods Harvested	Seeds Harvested	PCR Assayed Seeds	PCR+ Seeds (Positive Seeds %)	PCR Assayed Seedlings	PCR+ Seedlings (Positive Seedlings %)
ZH1160	251	293	73	54 (73.9)	50	9 (18.0)
ZH1105	462	488	122	66 (54.1)	60	46 (76.7)
ZH1059	364	350	88	61 (69.3)	60	8 (13.3)
ZH1085	585	733	183	72 (39.3)	60	30 (50.0)
ZH1071	398	570	143	101 (70.6)	60	0 (0)
ZH1069	674	681	170	141 (82.9)	60	0 (0)
Total	2734	3115	625	495 (79.2)	350	93 (26.6)

Note: The number of genomic DNA PCR positive seeds/seedlings out of the total number of seeds/seedlings PCR assayed is presented in parentheses as percentages.

The seed PCR products were gel resolved (Figure 2), producing a band of 699 bp, which is consistent with the expected size. The band was subsequently recovered and sequenced by the Sanger method (Comate Bioscience, Changchun, China). NCBI-BLAST was used to verify their identity. The sequenced bands were found to be identical to the mRNA of *Glycine max* C_2H_2 zinc-finger protein gene *SCTF-1* partial coding sequence (GenBank accession JQ692081).

Out of the 625 T_0 seeds, 495 seeds tested positive, resulting in a positivity rate of 79.2%. Specifically for each landrace, the PCR positive rate ranged from 39.3% (ZH1085) to 82.9% (ZH1069) (Table 3).

The positive seeds were planted, resulting in healthy T_1 seedlings. A total of 350 seedlings grown from genomic DNA PCR positive seeds were PCR-assayed using primers specific to *Bar*, *NOS*, and *SCTF-1* genes to reaffirm the presence of the pCPB-SCTF-1 construct (Figures 1 and 2). The peanut seedlings that tested positive by PCR produced amplification products of the expected sizes: 552 bp with the *Bar* primer pair, 250 bp with the *NOS* primer pair, and 699 bp with the *SCTF-1* primer pair (Figure 1).

In total, 93 seedlings tested positive, leading to a positivity rate of 26.6%. Regarding the recipients, the PCR positive rate varied significantly, ranging from 0% (ZH1071 and ZH1069) to 76.7% (ZH1105) (Table 3). Notably, although the two genotypes ZH1071 and ZH1069 tested positive in the T_0 generation, they were negative in the T_1 generation when assessed by genomic DNA PCR; therefore, they were excluded from further analysis (Table 3).

3.3. RT-PCR and qRT-PCR Analysis of Genomic DNA PCR Positive Seedlings

Seedlings that tested positive in the PCR assay were subjected to chilling stress at 5 °C for 95 h to study the expression of the *SCTF-1* transgene, and their responses were observed and compared to those of the untransformed control (CK).

RNA was extracted from the leaves, stems, and roots of both CK and putative transgenic seedlings. The presence of *SCTF-1* at the RNA level was confirmed by reverse transcription PCR analysis (Figure 3) and direct sequencing of RT-PCR products (Figure 4).

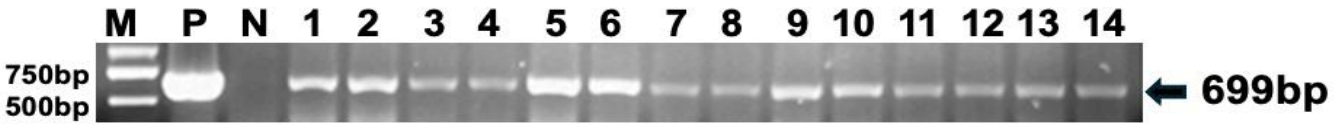

Figure 3. RT-PCR analysis of transgenic peanut seedlings using *SCTF-1* gene specific primers. M: 2000 bp DNA marker; P: positive control; N: untransformed negative control; 1–14: putative transgenic seedlings derived from ZH1160, ZH1105, ZH1059 and ZH1085.

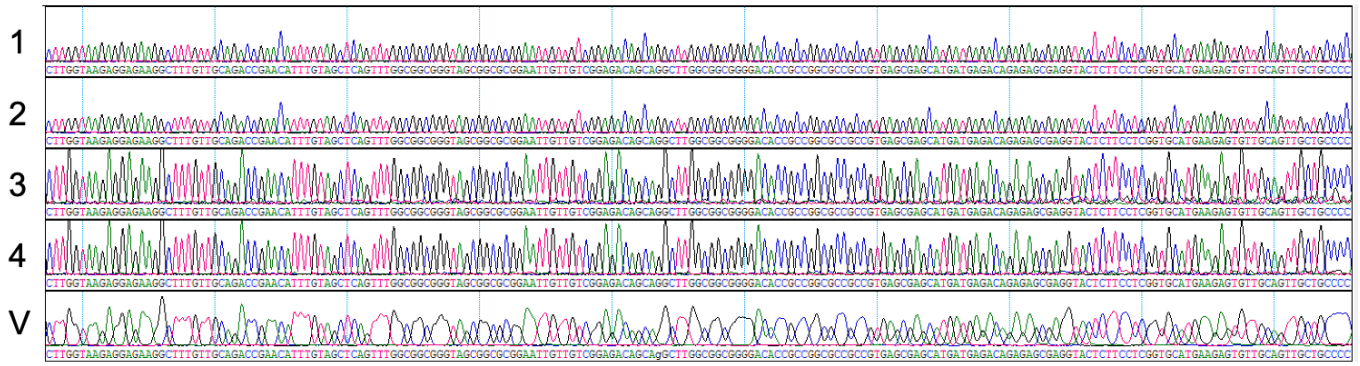

Figure 4. Comparison of chromatograms: partial sequences from direct sequencing of RT-PCR products from seedlings (1–4) aligned with the corresponding *SCTF-1* gene sequence region in the vector (V).

The expression levels were further quantified using qRT-PCR comparative analysis with cDNA from T_1 seedlings grown under both chilling stress and normal temperature conditions (Figure 5).

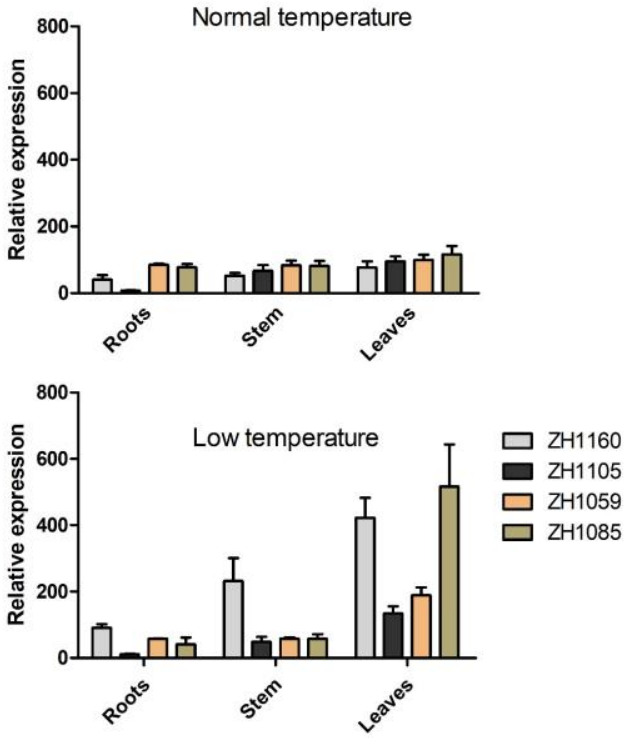

Figure 5. Relative expression of *SCTF-1* at mRNA level as determined by qRT-PCR in RT-PCR positive seedlings at normal temperature and at low temperature (5 °C for 95 h), with error bars indicating the standard error of the mean.

The results of ANOVA and multiple comparisons demonstrated that, overall, low-temperature treatment significantly enhanced the expression of *SCTF-1* transgene ($p < 0.01$). Among the genotypes, ZH1160 and ZH1085 exhibited significantly higher expression levels than ZH1105 ($p < 0.01$), while ZH1059 showed intermediate expression. Expression levels in leaf tissues were significantly higher than those in stem and root tissues ($p < 0.01$). However, the specific responses varied by genotype and tissue type.

For ZH1160, low-temperature treatment significantly increased expression compared to normal temperature ($p < 0.01$), with the expression ratio of leaf tissue under low vs. normal temperature reaching 5.48. Leaf tissue exhibited significantly higher expression than root tissue ($p < 0.05$), with the highest expression observed in leaf tissue under low-temperature treatment (422.58), which was significantly greater than all other combinations of temperature and tissue type ($p < 0.05$).

For ZH1085, low-temperature treatment also resulted in significantly higher expression compared to normal temperature ($p < 0.05$), with a low-to-normal temperature expression ratio of 4.43 in leaf tissue. Expression in leaf tissue was significantly higher than in stem and root tissues ($p < 0.01$), with the highest expression recorded in leaf tissue under low-temperature treatment (517.19).

For ZH1059, low-temperature treatment significantly enhanced expression compared to normal temperature ($p < 0.01$), with the leaf tissue expression ratio under low vs. normal temperature being 1.9. Leaf tissue exhibited significantly higher expression than stem and root tissues ($p < 0.01$), with the maximum expression level observed in leaf tissue under low-temperature treatment (189.21).

For ZH1105, expression in leaf tissue was significantly higher than in stem and root tissues ($p < 0.01$).

These results highlighted the consistent upregulation of *SCTF-1* transgene under low-temperature conditions, particularly in leaf tissues, with notable genotype-specific differences in expression levels and responses to temperature treatments.

3.4. Southern Blot Analysis

The integration of the *SCTF-1* gene into the peanut genome of four genomic DNA PCR positive seedlings derived from the four landraces (ZH1160, ZH1105, ZH1059, and ZH1085) was detected using the Southern blot technique. Hybridization signals were detected only in ZH1160- and ZH1085-derived seedlings (Figure 6), while no bands were observed for those derived from ZH1105 and ZH1059 (Figure 6).

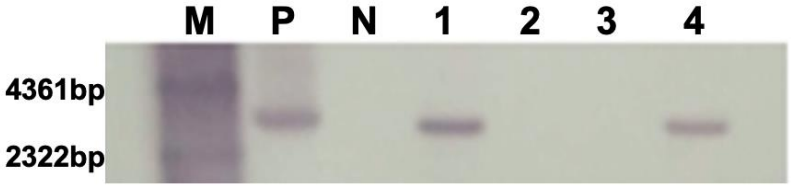

Figure 6. Southern blot analysis of putative transgenic peanut seedlings. M: DIG DNA marker; P: positive control (plasmid DNA uncut); N: untransformed negative control; 1–4 representing ZH1160, ZH1105, ZH1059, and ZH1085 derived genomic DNA PCR positive seedlings, respectively.

3.5. Assessment of Chilling Stress Tolerance in Transgenic and Control Seedlings

During the germination stage and the first three weeks of growth under fluorescent lights in the growth chamber, no phenotypic differences were observed between the transgenic seedlings and their respective controls. To evaluate whether SCTF-1 transgenic plants show enhanced tolerance to chilling stress, a low-temperature stress experiment was conducted. The growing conditions for both transgenic and control seedlings were altered from 28 °C/22 °C (day/night) to a constant 5 °C (day/night) with a 12-h photoperiod.

After 95 h of exposure to 5 °C, clear phenotypic differences were observed between the transgenic seedlings and their controls (Figure 7). Untransformed control plants exhibited visible chlorophyll loss and more severe wilting compared to the transgenic plants.

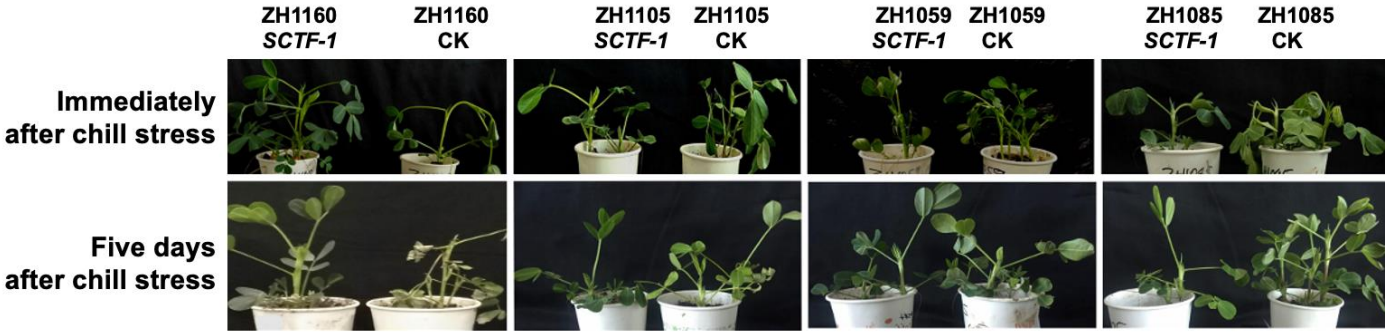

Figure 7. Phenotypes of transgenic peanut seedlings immediately and five days after chilling stress treatment. Landraces labeled with *SCTF-1* represent transgenic seedlings, while those labeled with CK represent untransformed controls.

Following the chilling treatment, the recovery of both transgenic and control plants was monitored at room temperature (approximately 25 °C) for two to five days. All transgenic seedlings recovered within two days and were thus classified as highly tolerant to chilling stress (Figure 7). In contrast, the untransformed control plants required more time to recover and exhibited varying levels of sensitivity to chilling stress (Figure 7). The control plants of ZH1160 took more than five days to recover, losing most of their leaves (both basal and cauline), and were classified as highly sensitive. The control plants of ZH1105 recovered after five days but lost some basal leaves. Meanwhile, the controls of ZH1059 and ZH1085 retained most of their leaves and were considered sensitive.

These phenotypic differences clearly demonstrated a significant disparity in the ability to withstand chilling stress between the transgenic seedlings and the untransformed control plants.

3.6. Biochemical Assay of Peanut Landraces and Their Transformants

Three biochemical indicators were analyzed to investigate the mechanisms underlying the phenotypic responses of the transgenic seedlings. The results are presented in Table 4.

In terms of proline content, under normal temperature conditions, among the four combinations of landraces and their corresponding transgenic seedlings, only one combination showed a significant difference at 0.05 level, with ZH1105 SCTF-1 exhibiting higher proline levels than its non-transgenic control. After low-temperature treatment, three out of the four combinations demonstrated significantly higher proline content in the transgenic seedlings compared to their non-transgenic counterparts. The proline content in transgenic peanuts showed an increasing trend following low-temperature treatment, while the non-transgenic controls exhibited little difference compared to normal temperature conditions. These results indicated that *SCFT-1* transgenic plants generally accumulated higher levels of proline under chilling stress, suggesting enhanced osmotic adjustment and improved stress tolerance.

Regarding MDA content, there were no significant differences between transgenic seedlings and their non-transgenic controls under normal temperature conditions across all four combinations. However, after low-temperature treatment, MDA levels in transgenic seedlings were significantly lower than those in the controls at 0.05 level. Compared to normal temperature conditions, the increase in MDA levels for transgenic seedlings after low-temperature treatment was relatively small, while the non-transgenic controls exhibited

a much larger increase. These results suggested that *SCFT-1* transgenic plants experienced reduced lipid peroxidation under chilling stress, indicating enhanced membrane stability.

With respect to POD activity, under normal temperature circumstances, in only one (ZH1160 SCTF-1 and ZH1160) out of the four combinations, transgenic seedlings possessed a significantly higher activity than their non-transgenic controls at 0.05 level. After low-temperature treatment, two combinations (ZH1160 SCTF-1 and ZH1160, ZH1059 SCTF-1 and ZH1059) manifested a notably higher POD activity in transgenic seedlings in comparison to their non-transgenic counterparts at the same significance level. These findings indicated that *SCFT-1* transgenic plants exhibit higher POD activity under chilling stress, contributing to enhanced antioxidant capacity.

Table 4. Comparison of three biochemical indicators in peanut landraces and their transformants under normal and low-temperature conditions.

Biochemical Indicator	Landrace and Its Transformant	Normal Temperature (28 °C/22 °C)	Low Temperature 5 °C (95 h)
Proline (μmol/g)	ZH1160 SCTF-1	3.4230 a	4.2279 a
	ZH1160 CK	3.0113 a	3.1546 b
	ZH1105 SCTF-1	3.0650 a	3.3440 a
	ZH1105 CK	2.3293 b	2.5214 b
	ZH1059 SCTF-1	2.7107 a	3.9588 a
	ZH1059 CK	2.5453 a	2.6495 b
	ZH1085 SCTF-1	2.6749 a	2.8304 a
	ZH1085 CK	2.3043 a	2.2134 a
MDA (nmol/mL)	ZH1160 SCTF-1	0.2256 a	0.2570 b
	ZH1160 CK	0.2419 a	0.4591 a
	ZH1105 SCTF-1	0.1731 a	0.2591 b
	ZH1105 CK	0.1798 a	0.5931 a
	ZH1059 SCTF-1	0.2142 a	0.2787 b
	ZH1059 CK	0.2794 a	0.4445 a
	ZH1085 SCTF-1	0.1757 a	0.2376 b
	ZH1085 CK	0.1794 a	0.4482 a
POD [U/(g·min)]	ZH1160 SCTF-1	64.3506 a	65.9530 a
	ZH1160 CK	55.8336 b	59.3420 b
	ZH1105 SCTF-1	61.6116 a	61.7890 a
	ZH1105 CK	61.3386 a	61.4610 a
	ZH1059 SCTF-1	63.0360 a	66.6320 a
	ZH1059 CK	62.0196 a	63.2420 b
	ZH1085 SCTF-1	62.0760 a	64.4270 a
	ZH1085 CK	60.6732 a	62.3010 a

Note: For each biochemical parameter and for each peanut landrace and its corresponding transformant, values marked with the same letter were not significantly different at the 0.05 level. Landraces labeled with SCTF-1 represent transgenic seedlings, while those labeled with CK represent untransformed controls.

4. Discussion

4.1. Utility of Nodal Injection of A. tumefaciens in Functional Analysis of Genes

In an unpublished study, our team utilized a previously identified peanut disease resistance gene, *NPR1* [27], and the bacterial wilt-responsive genes *CYP* and *ARF* [28] to construct overexpression and antisense expression vectors. Overexpression vectors were introduced into the bacterial wilt-susceptible cultivar Huayu 40, while antisense expression vectors were transferred into the bacterial wilt-resistant cultivar Rihua 1 using the nodal Injection method. This transformation effectively reversed their respective disease responses in T_1 plants. The expression levels of the target genes were assessed, ELISA was performed, and the roles of the three genes in bacterial wilt disease resistance were confirmed. However, Southern blot analysis was not conducted.

Chilling stress during the seedling stage disrupts photosynthesis, reduces nutrient uptake, and increases oxidative damage, leading to stunted growth and yield loss [29,30]. Enhancing peanut chilling tolerance is a cost-effective solution. In the present study, we demonstrated that the use of nodal injection in the transfer of the *SCTF-1* gene, which encodes a C_2H_2-type zinc finger protein in soybean, significantly enhanced chilling tolerance in transgenic peanut. This result is consistent with previous studies in transgenic tobacco and soybean plants [16,31]. qRT-PCR analysis revealed differential expression of the *SCTF-1* gene across various plant parts in transgenic seedlings. Under low-temperature conditions, *SCTF-1* expression was significantly upregulated in the transgenic seedlings compared to those grown under normal conditions. These results were corroborated by phenotypic observations. Biochemical analysis further supported the role of *SCTF-1* in enhancing chilling tolerance. The outcome from the present study demonstrated that *SCFT-1* transgenic peanut plants exhibited improved cold stress tolerance through increased proline accumulation, reduced lipid peroxidation, and elevated POD activity. The higher proline levels suggested better osmotic adjustment under stress conditions, while the lower MDA content indicated reduced oxidative membrane damage. Furthermore, the increased POD activity reflected a strengthened antioxidant defense system, which likely mitigated reactive oxygen species (ROS)-induced damage. These findings aligned with previous studies on *SCTF-1* transgenic soybean [16] and suggested that *SCFT-1* played a critical role in enhancing the physiological resilience of peanut to cold stress, providing a valuable strategy for improving cold tolerance in peanut breeding. More importantly, this study provided evidence of *SCTF-1* gene integration through Southern blot analysis.

Taken together, the results from molecular biology assays, seedling cold treatment-induced trait changes, and biochemical analyses demonstrated the successful transformation of peanut via nodal injection. In this study, as well as in the above-mentioned research on bacterial wilt resistance, the roles of the introduced genes were evident as early as the T_1 generation, further confirming that nodal injection is a viable tool for gene function analysis.

4.2. Possible Reasons for Discrepancy Between Seed and Seedling PCR Results

The discrepancy between genomic PCR results obtained from seeds and their corresponding seedlings is a well-documented phenomenon in *Agrobacterium*-mediated transformation studies, and this issue was also observed in the present study. Specifically, while some seeds yielded positive genomic PCR results, their corresponding seedlings failed to amplify the transgene (Table 3), indicating a potential loss or instability of the T-DNA.

One plausible explanation is the transient presence of T-DNA during the transformation process. Non-integrated T-DNA may persist as extrachromosomal DNA within the seed tissues, producing a positive PCR signal. However, this extrachromosomal DNA is often degraded or diluted during germination, resulting in the absence of detectable T-DNA in the seedlings.

Another contributing factor may involve the chimeric composition of seeds. In peanut seeds, distinct tissues, such as cotyledonary tissues and embryonic (germ) tissues, coexist. The positive PCR signal may originate from T-DNA localized in the cotyledonary tissues or other non-heritable structures. In this study, to avoid impacting seed germination, DNA for seed genomic PCR analysis was exclusively extracted from the cotyledonary tissues, while the immature leaflet tissue (which develops into true leaves) was not sampled. Conversely, DNA from seedlings was extracted from true leaf tissues. If transformation occurs only in the cotyledonary tissues without successful integration into the germ tissue, this discrepancy between genomic PCR results from seeds and seedlings could arise.

4.3. Possible Reasons for Genomic DNA PCR-Positive but Southern Blot-Negative Results

In this study, discrepancies were observed, in that genomic DNA PCR-positive samples yielded negative Southern blot results (Table 3, Figure 6). This phenomenon highlights the distinct sensitivities and specificities of these two techniques.

Genomic DNA PCR is an exceptionally sensitive method that is capable of amplifying minute quantities of DNA, including fragmented, extrachromosomal, or incomplete T-DNA sequences that have not been stably integrated into the genome. In contrast, Southern blotting detects T-DNA integration through hybridization and requires sufficient signal intensity for visualization. When T-DNA is integrated at low copy numbers, is rearranged, or only partially integrated, the hybridization signal may fall below the detection threshold of the Southern blot, leading to a negative result.

Furthermore, it is still possible that the digested fragments of genomic DNA are too large to be effectively separated by gel electrophoresis. This may result in false negatives in Southern blotting.

4.4. Future Research

Notably, in this study, the transgenic lines carrying the *SCTF-1* gene were driven by the constitutive 35S promoter. However, under low-temperature conditions, an upregulation of expression was observed compared to normal temperatures, at least in certain genotypes. A similar finding was also recorded in soybean [17]. This phenomenon may result from changes in transcription factor expression/activity, RNA polymerase stability, and mRNA stability. The underlying mechanisms behind these observations require further investigation. In this study, qRT-PCR sampling was destructive; thus, different plants were used for normal and low-temperature conditions. However, different plants may represent distinct transformation events. Nevertheless, since transgenic seedlings derived from four landraces were used in this study, the results still have a certain degree of credibility. The observed upregulation of expression under low-temperature treatment requires further evidence using stable transgenic lines.

For T_1 plants that were Southern blot-negative, or not assayed by Southern blot analysis, multi-generational validation is still needed to determine whether the transgene is present and stably inherited. Using other restriction enzymes that cut within the insert, such as *Eco*RI, is absolutely necessary. For Southern blot-positive plants, the inheritance of the transgene and low-temperature tolerance trait in subsequent generations has yet to be studied. Additionally, how *SCTF-1*-transformed peanut responds to low-temperature stress during the sowing and late growth stages is of particular interest. Furthermore, evaluating the agronomic performance of the transformants, especially in terms of pod and seed yields, under both optimal and stress conditions is crucial for gaining insights into their practical utility.

In this study, Southern blot analysis confirmed the stable integration of exogenous genes into the peanut genome. The result highlights the potential of nodal agroinjection to generate stable transgenic lines, establishing a solid foundation for improving peanut varieties through genetic engineering.

While functional analysis of plant genes is typically conducted in model plants such as *Arabidopsis thaliana*, certain genes—such as those involved in peanut pod development—are not well-suited for analysis in heterologous systems, making peanut the preferred system for such studies. In such cases, a highly efficient and user-friendly peanut transformation system is essential.

The advantages of this transformation method include low seed requirements and high seed yield, as well as simplicity and labor efficiency in the genetic transformation

process. Future studies should consider using constructs with fluorescent markers to reduce the workload involved in screening [32].

Peanut omics has advanced rapidly in recent years, yielding extensive datasets. For example, transcriptomic analyses have uncovered numerous differentially expressed genes, and many QTLs have been successfully mapped. However, a considerable number of studies ended at identifying candidate genes, without proceeding to experimental validation of their functions. This approach is expected to expedite functional genomics research in peanut and drive the molecular improvement of the peanut crop.

As reviewed by Bélanger et al., in planta stable transformation in plants primarily targets germlines as well as shoot apical and adventitious meristems [11]. Notably, the grafting-mediated transformation method has emerged as a novel and promising approach, with successful applications in other plant species [11]. This method warrants exploration in peanut as well, given its amenability to grafting. Recently, Song et al. provided a comprehensive overview of advancements in genetic transformation research in peanut [33]. Comparative studies on different transformation methods are also needed to optimize strategies for this crop.

5. Conclusions

In this study, the soybean cold tolerance transcription factor gene *SCTF-1* was successfully introduced into peanut using the innovative nodal agroinjection method. Genomic DNA PCR analysis, RT-PCR validation, and sequencing of RT-PCR products identified positive seedlings grown from PCR-positive seeds. *SCTF-1* expression in transgenic seedlings under low temperatures and normal temperatures was confirmed by qRT-PCR. Southern blot analysis provided conclusive evidence that *SCTF-1* had been stably integrated into the peanut genome. Compared to non-transgenic controls, transgenic peanut seedlings showed improved chilling tolerance and exhibited increased proline content and peroxidase (POD) activity, along with lower malondialdehyde (MDA) levels.

In summary, this study, from molecular, biochemical, and morphological perspectives, verified the feasibility of nodal agroinjection in peanut for functional gene analysis. Introducing the *SCTF-1* gene into peanut significantly enhanced the cold tolerance of peanut at the seedling stage.

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