Brief Report

Enhancing Agrobacterium-Mediated Hairy Root Transformation Efficiency in Peanut Through the Application of GRF, GIF and WOX Genes

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Enhancing Agrobacterium-Mediated Hairy Root Transformation Efficiency in Peanut Through the Application of GRF, GIF and WOX Genes

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Abstract

Peanut (*Arachis hypogaea* L.) is a major oil and economic crop, yet genetic transformation remains inefficient and time-consuming, hindering functional genomics and molecular breeding. Agrobacterium rhizogenes-mediated hairy root transformation provides a fast, stable, and low-cost platform for rapid testing of expression vectors, promoters, and CRISPR constructs, but its performance in peanut is often limited by low induction efficiency, small root biomass, and high variability among explants. Here, we identified multiple peanut Growth-Regulating Factor (*GRF*) genes, GRF-Interacting Factor (*GRF-GIF*) fusion genes and WUSCHEL-related homeobox (*WOX*) genes, constructed high-expression vectors, and delivered them into A. rhizogenes to infect peanut stem segments. Relative to the empty-vector control, expression of these developmental regulators markedly enhanced hairy-root induction and growth: the number of roots per explant increased by 1.3–2.4-fold, and the resulting roots were thicker and more highly branched. GUS staining confirmed stable transgene expression in induced roots. Collectively, these results improve the efficiency of peanut hairy-root systems and provide a practical toolset for rapid functional validation, promoter evaluation, CRISPR activity testing, and metabolic engineering.

Keywords: hairy roots; *GRF*; *GIF*; *WOX*

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important oil crop cultivated in more than 100 countries [1]. Their seeds are rich in lipids, proteins, folate, tocopherols, phytosterols, and polyphenols [2,3]. With rapid advances in biotechnology, genetic engineering has become a powerful approach for crop improvement [4,5]. However, peanut transformation still suffers from low efficiency and long regeneration cycles, limiting functional gene studies and molecular breeding [6]. Agrobacterium rhizogenes-induced hairy roots are fast-growing roots generated after infection and have been widely used for gene function studies, secondary metabolite production, and trait improvement [7]. In peanut, the system is still constrained by low induction efficiency, insufficient root biomass, and large variability among explants.

Growth-Regulating Factors (*GRFs*) are plant-specific transcription factors that promote growth and organ development. GRF-Interacting Factors (*GIFs)* act as transcriptional co-regulators and are essential for cell proliferation, organogenesis, and regeneration [8,9]. Overexpressing *AtGRF1* or *AtGRF2* in *Arabidopsis* increases leaf size, and overexpression of the pear gene *PbGRF18* in tomato can promote fruit development and sugar accumulation [10,11]. Notably, *GRF-GIF* fusion (chimera) constructs can enhance transformation and regeneration efficiency and shorten regeneration cycles in crops such as maize, wheat, and soybean [12–14]. In peanut, however, the application of *GRF*/*GIF* has

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rarely been reported, highlighting the need to evaluate *GRF-GIF* tools and develop a more efficient and practical transformation platform.

WOX proteins are key transcription factors that maintain meristem activity and regulate organogenesis by controlling cell fate and re-differentiation [15]. In *Arabidopsis*, the *WOX* family (*WUS* and *WOX1-WOX14*) participates in development of multiple organs, including roots, stems, leaves, flowers, and fruits [16]. *WOX11* directly activates *LBD16* to initiate root primordia and thereby promotes lateral and adventitious root formation [17]. In woody species, *BpWOX11* (Betula platyphylla) increases adventitious rooting of cuttings, and *JrWOX5* (walnut) promotes root formation and influences plant architecture [18,19]. Despite these advances, the use of *WOX* genes to enhance induction performance in peanut hairy-root systems remains limited, and a *WOX*-based tool that improves rooting efficiency would be valuable.

In this study, we identified and cloned multiple peanut *GRF* genes, *GRF-GIF* fusion genes and *WOX* genes, and constructed high-expression vectors for peanut hairy-root transformation. With the exception of 2S-PL-GUS and GRF-2A-T-GUS, which showed lower transformation efficiency than the empty-vector control, most constructs improved transformation efficiency, with GRF-2A(396)-GIF-GUS achieving the highest rate (85.14 ± 2.94%; Table 1). Compared with the empty-vector control, GRF, GIF, and WOX treatments also promoted hairy-root growth, increasing the number of roots per explant by 1.3–2.4-fold and producing thicker, more highly branched roots. These tools can strengthen peanut hairy-root platforms for rapid gene function validation, trait improvement, secondary metabolite production, and related applications.

Table 1. Root regeneration and transformation rates recorded after injection.

Constructs	Number of regenerated roots per explant after one week	Number of regenerated roots per explant after 2 weeks	Number of regenerated roots per explant after 3 weeks	Number of regenerated roots per explant after 4 weeks	Transformation rate %
GRF-V829-T-GUS	1.67±0.58c	6.33±0.58d	21.00±1.00d	34.00±1.00b	67.69±2.00c
2S-PL-GUS	1.00±0.00cd	6.33±1.53d	17.67±1.15e	25.00±1.73d	60.20±4.35de
GRF-2A(396)-GIF-GUS	6.33±1.53a	16.33±1.53a	36.33±0.58a	44.67±1.53a	85.14±2.94a
GRF-2A-GIF-GUS	3.67±0.58b	8.67±0.58c	23.67±1.53c	32.00±1.00bc	78.18±2.45b
GRF-FF-GIF-GUS	3.00±1.00b	10.33±0.58b	25.67±0.58b	31.33±1.53c	76.72±3.69b
GRF-2A-T-GUS	0.67±0.58cd	6.67±0.58d	17.33±0.58e	27.00±1.00d	55.61±2.06f
PLVV0P-T-GUS	1.33±0.58cd	5.67±0.58d	15.00±1.00f	27.00 1.00d	70.44±2.61c
pCAMBIA1381-AhUBQ4-GUS	0.00±0.00d	2.67±0.58e	11.67±0.58g	18.67±1.53e	64.58±5.44cd

2. Results

2.1. Phylogenetic Selection of Peanut GRF/GIF/WOX Candidates and Construction of Expression Vectors

To identify peanut developmental regulators with potential utility in transformation enhancement, we performed phylogenetic analyses of GRF, GIF and WOX proteins from peanut and representative model/crop species. In the *GRF* clade, several peanut proteins grouped closely with established regulators such as *AtGRF5* and *TaGRF4*, supporting functional conservation and motivating the selection of *GRF-V829EQ*,*GRF-2A7ZAY* and *GRF-FF6C67* for downstream testing (Figure 1). Similarly, the peanut candidate *GIF-HK1F5C* clustered with the reference *OsGIF*, indicating it represents a likely functional *GIF* ortholog (Figure 1). For the *WOX* family, *WOX-PLVV0P* and an additional candidate, *WOX-ZS5XSZ* grouped with the reference *AtWOX5*, suggesting conserved roles associated with meristematic or regenerative competence (Figure 1).

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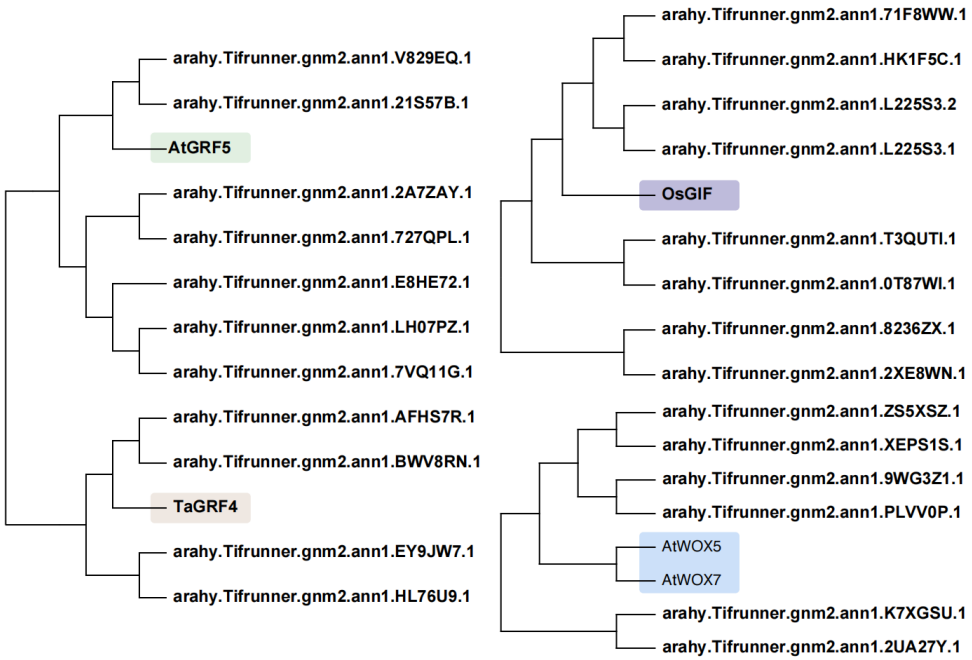

Figure 1. Phylogenetic relationships of representative *GRF*, *GIF* and *WOX* proteins from Arachis hypogaea cv. Tifrunner and reference species (*Arabidopsis*, rice, and wheat). Protein sequences were aligned and used to infer the tree. Tifrunner genes are labeled with their genome annotation IDs (arahy.Tifrunner.gnm2.ann1.*). Candidate regulators selected for vector construction and functional testing are highlighted, including *GRF-V829EQ*, *GRF-2A7ZAY* and *GRF-FF6C67*; *GIF-HK1F5C*; *WOX-PLVV0P* and *WOX-ZS5XSZ*), alongside known reference regulators (e.g., *AtGRF5*/*TaGRF4*, *OsGIF*, *AtWOX5*).

Based on these phylogenetic relationships, we generated a compact vector set to evaluate single and combinatorial regulator modules in peanut hairy-root assays. All constructs shared a common architecture including a *35S*::HygR selection cassette and a *pAhUBQ4*::GUS reporter cassette for rapid quantification of transformation output (Figure 2). Regulator expression cassettes were driven by *pAhUBQ4*, including single-gene constructs (*GRF-V829EQ*, *GRF-2A7ZAY*, *WOX-PLVV0P*) as well as combinatorial modules, notably GRF-GIF co-expression constructs (*GRF-2A7ZAY–GIF-HK1F5C* and *GRF-FF6C67–GIF-HK1F5C*) and a miR396-modified *GRF-GIF* module to mitigate miR396-associated repression (Figure 2). Together, these designs established a standardized genetic toolkit for comparing developmental regulator modules under the same promoter and reporter framework (Figures 1 and 2).

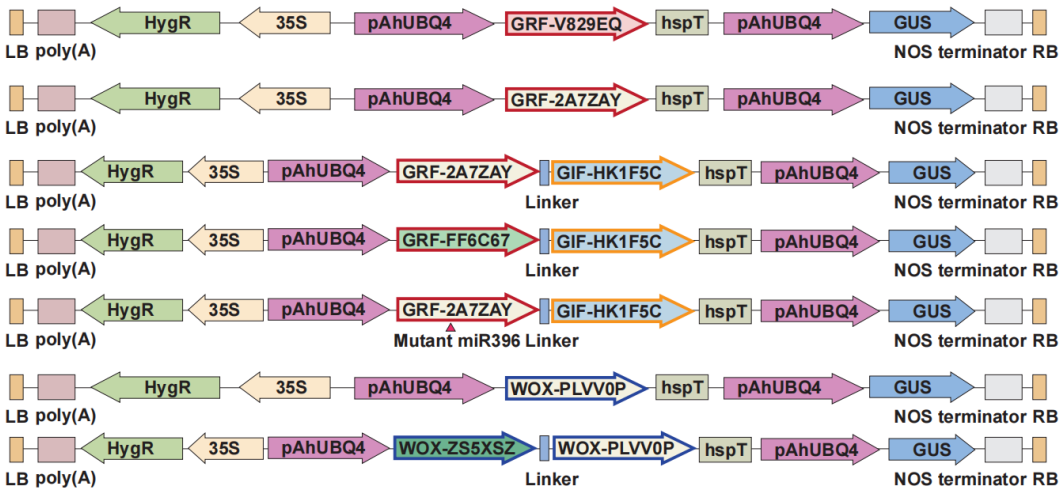

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Figure 2. Schematic representation of binary T-DNA vectors used for peanut hairy-root transformation assays. All constructs contain a *35S*-driven hygromycin resistance marker (Hyg) for selection and a *pAhUBQ4*-driven GUS reporter followed by a *NOS* terminator for scoring transformed roots. Developmental regulator (*DR*) expression cassettes were driven by pAhUBQ4 and include single-gene constructs (e.g., *GRF-V829EQ*, *GRF-2A7ZAY*, *WOX-PLVV0P*), combinatorial modules for co-expression of GRF and GIF (linked by a 2A peptide, as indicated), a miR396-modified *GRF* module (denoted "Mutant miR396") assembled with *GIF-HK1F5C*, and a dual-*WOX* construct (*WOX-ZS5XSZ–WOX-PLVV0P*) connected by a linker. LB, left border; RB, right border.

2.2. Induction of Hairy Roots by GRF, GIF and WOX Genes

In our preliminary work, we established a peanut hairy-root transformation system. Using 30-day-old rootless seedlings as explants, *Agrobacterium rhizogenes* K599 carrying a GUS reporter and developmental regulator constructs was used to infect peanut stem segments. Driven by the peanut endogenous promoter *AhUBQ4*, transgenic hairy roots were efficiently induced (Figure 3) [20].

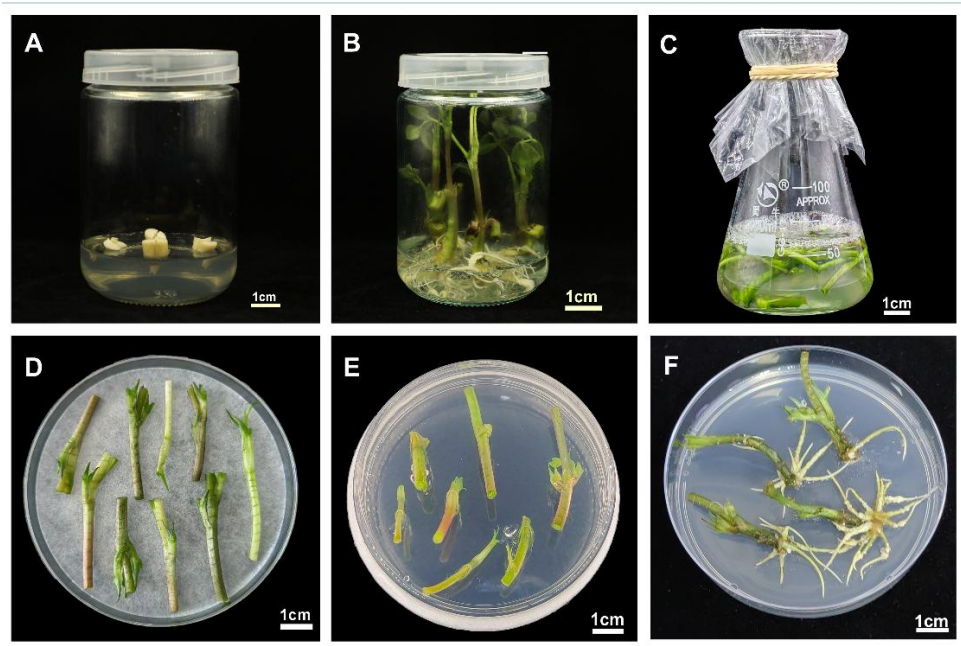

Figure 3. Transgenic *A. hypogaea* hairy roots obtained by *A. rhizogenes*-mediated transformation. (**A**):Seed germination. (**B**):Cultivate sterile seedlings for 30 days. (**C**):*A. rhizogenes* infection on explants. (**D**): Co-cultivation of explants after infection with *A. rhizogenes*. (**E**): Infused explants were transferred to induction culture medium. (**F**):The hairy roots produced.

Developmental regulators such as *GRF*, *GIF* and *WOX*—and their combinations—can improve transgenic efficiency and shorten regeneration cycles in multiple species [12,21,22]. To evaluate their effects in peanut, we quantified transformation performance and performed GUS staining in transgenic hairy roots. Except for 2S-PL-GUS and GRF-2A-T-GUS, which showed lower transformation efficiency than the empty-vector control, all other constructs improved transformation efficiency. Among them, GRF-2A(396)-GIF-GUS showed the highest transformation efficiency (85.14 ± 2.94%; Table 1). Relative to the empty-vector control, *GRF*, *GIF* and *WOX* constructs also promoted root growth, increasing the number of hairy roots per explant by 1.3–2.4-fold and producing thicker, more highly branched roots (Table 1).

Explants from the same infection batch were transferred to MS medium and grown for 2 weeks before GUS staining. The staining results further supported that GRF, GIF and WOX constructs promote peanut hairy-root growth and branching (Figure 4).

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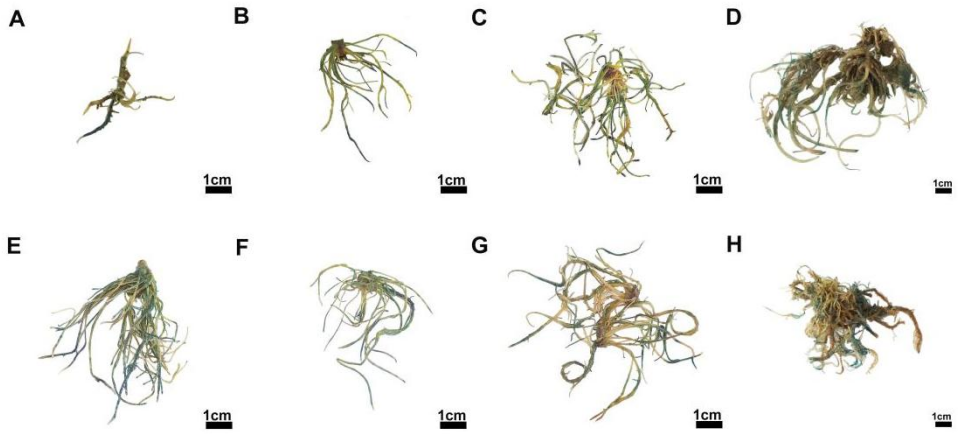

Figure 4. GUS staining images of hairy roots transformed by control and various carriers. (**A**): control, pCAMBIA1381-AhUBQ4-GUS. (**B**):2S-PL-GUS. (**C**):2S-PL-GUS. (**D**):GRF-2A(396)-GIF-GUS. (**E**):GRF-FF-GIF-GUS. (**F**):PLVV0P-T-GUS. (**G**):GRF-2A-GIF-GUS. (**H**):GRF-V829-T-GUS.

3. Discussion

Peanut is an allotetraploid legume formed through hybridization and domestication of the wild diploid ancestors *Arachis duranensis* (AA genome) and *Arachis ipaensis* (BB genome) [23,24]. As a major oilseed crop, peanut seeds contain abundant storage proteins [25]. Establishing an efficient genetic transformation system is critical for genetic engineering-based germplasm innovation. However, current peanut transformation methods often suffer from chimerism, low transformation efficiency, and poor regeneration efficiency, which constrain functional genomics and molecular breeding [26]. *Agrobacterium rhizogenes*-mediated hairy-root transformation integrates T-DNA from the Ri plasmid into the plant genome, inducing transgenic hairy roots [27]. Because it bypasses complex tissue culture and whole-plant regeneration, this approach enables rapid generation of composite plants with relatively short cycles and good genetic stability [28]. It is therefore well suited for root-specific gene function studies, metabolite production, and heterologous protein expression [29].

Overexpression of developmental regulators has broad utility for improving plant regeneration and transformation. *GRF-GIF* fusion expression can substantially enhance transformation efficiency, increasing rates by 3.0–4.7-fold in wheat, rye, tomato, and citrus [12,30]. *WOX* genes also influence root development and differentiation: for example, *WOX11* overexpression in rice increases adventitious root formation, whereas *WOX11* mutants show root defects [31]. Plants expressing *TaWOX9* in *Arabidopsis* develop longer roots than controls, indicating a role in promoting root growth [32]. Consistent with these reports, our peanut *GRF*, *GRF-GIF* and *WOX* constructs improved hairy-root induction and growth, increasing roots per explant by 1.3–2.4-fold and generating thicker, more highly branched roots. GUS staining confirmed stable transgene expression, supporting the use of these constructs as practical tools for peanut functional validation and metabolic engineering.

Although GRF-2A(396)-GIF-GUS produced transgenic hairy roots with high transformation efficiency and genetic stability, regenerating whole plants from hairy roots remains challenging. To date, stable transgenic plants regenerated from hairy roots have been reported for only a limited number of species, including sweet potato, radish, apple, and several medicinal plants [22,33,34]. Previous studies show that developmental regulators such as *WOX*, *GRF* and *GIF* play important roles in organ regeneration. For example, the *Wus2-ipt* combination can promote callus formation and directly induce regeneration buds at wound sites without exogenous hormones; using early-bolting genotypes, transgenic seeds can be obtained within 6-7 months, helping overcome radish transformation bottlenecks [22]. In sugar beet, *AtGRF5* promotes shoot formation and improves

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transformation of recalcitrant varieties, and *GRF5*/*GRF6*/*GRF9* can induce callus formation and differentiation in rapeseed [35]. *GRF-GIF* chimeras in Arabidopsis, wheat, rice, and maize enhance regeneration, potentially through activation of transcription via interactions with *SWI2*/*SNF2* complexes [12,36]. Because GRF and GIF proteins are highly conserved, GRF-GIF chimeras may be broadly applicable to species with low regeneration efficiency [9]. Our results demonstrate that these developmental regulators can improve peanut hairy-root transformation and growth; future work should test their utility in peanut regeneration from hairy roots.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

We followed previously published protocols with modifications. Seeds from four red-seeded peanut varieties were surface-rinsed and soaked overnight. After removing the seed coat under a laminar-flow hood, seeds were surface-sterilized in 75% ethanol for 1 min and then in 2.5% sodium hypochlorite (available chlorine) for 15 min. Seeds were rinsed five times with sterile water. Half of each cotyledon was removed with a scalpel, and cotyledons with intact embryos were placed on MS medium for germination. Seedlings were grown in a tissue-culture room under a 28 °C day/25 °C night cycle with 16 h light/8 h dark (Figure 3A,B).

4.2. Identification and Cloning of GRF, GIF and WOX Genes in Peanut

Candidate members of the *GRF*, *GIF* and *WOX* gene families were identified from the *Arachis hypogaea* cv. Tifrunner genome (https://data.legumeinfo.org/Arachis/hypogaea/genomes/Tifrunner.gnm2.J5K5/) [37]. Protein sequences of reported GRF/GIF/WOX regulators from *Arabidopsis*, wheat, and rice were used as queries for homology-based searches against the peanut proteome. Putative candidates were further validated by confirming the presence of conserved domains using standard domain databases (NCBI CDD).

To infer evolutionary relationships and support candidate selection, representative GRF/GIF/WOX proteins from peanut and reference species were aligned and used to construct phylogenetic trees (Figure 1). Based on phylogenetic placement relative to known regulators (*AtGRF5*, *TaGRF4*, *OsGIF*, *AtWOX5*), several peanut candidates were prioritized for cloning and functional evaluation, including *GRF-V289EQ*, *GRF-2A7ZAY*, *GRF-FF6C67*, *GIF-HK1F5C*, *WOX-PLVV0P* and *WOX-ZS5XSZ* (Figures 1 and 2).

Total RNA was extracted from young peanut leaves (Tifrunner) and reverse-transcribed into cDNA. Full-length coding sequences (CDSs) were amplified using gene-specific primers (Table S1), purified, cloned into an intermediate vector, and verified by Sanger sequencing prior to binary vector assembly.

4.3. Vector Construction

Binary vectors were constructed using a backbone reported previously [20]. As illustrated in Figure 1B, all constructs were designed within the T-DNA region and included: (i) a hygromycin resistance cassette (Hyg) driven by the *CaMV35S* promoter for selection, and (ii) a GUS reporter cassette driven by the *AhUBQ4* promoter (*pAhUBQ4*) to facilitate rapid scoring of transformed hairy roots.

For functional testing of developmental regulators, individual CDSs and combinatorial modules were expressed under *pAhUBQ4* (Figure 2), including *GRF-V829EQ*, *GRF-2A7ZAY*, *WOX-PLVV0P*, and a dual-*WOX* construct (*WOX-ZS5XSZ-WOX-PLVV0P*) connected by a linker. To enable co-expression of *GRF* and *GIF*, *GRF-GIF* combinatorial constructs were generated by linking *GRF-2A7ZAY* (or *GRF-FF6C67*) with *GIF-HK1F5C* using a linker sequence (Figure 2). In addition, a miR396-related modified GRF module (as indicated by "Mutant miR396" in Figure 2) was generated to reduce

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miR396-mediated repression while maintaining the encoded protein sequence, and then assembled with GIF-HK1F5C for co-expression.

All plasmids were verified by restriction digestion and sequencing, and then introduced into *Agrobacterium rhizogenesstrain* K599 using standard procedures. Transformed strains were selected on appropriate antibiotics before infection assays.

4.4. Hairy Root Transformation

Stem segments (3–5 cm) from in vitro–grown seedlings were wounded with a scalpel and infected with *Agrobacterium rhizogenes*. For infection, *A. rhizogenes* was resuspended in infection medium (1/2 MS liquid medium supplemented with 100 μM acetosyringone) and adjusted to OD600 = 0.6; the suspension was incubated at 28 °C for 0.5–2 h. Explants were soaked in the infection suspension and shaken at 150 rpm for 30 min (Figure 3C). After infection, explants were washed 2–3 times with sterile water, blotted dry, and placed on 1/2 MS solid medium over filter paper for co-cultivation at 23 °C for 3 days (Figure 3D). Explants were then transferred to 1/2 MS solid medium containing timentin (300 mg/L) to suppress *Agrobacterium* and induce hairy roots (Figure 3E). Cultures were maintained at 28 °C under 16h light/8h dark, and the medium was refreshed every 2 weeks (Figure 3F).

4.5. GUS Histochemical Staining

Infected hairy roots were incubated in GUS staining solution (GUS staining kit, COOLABER, SL7160) at 37 °C for 24 h. Samples were then rinsed 2–3 times with sterile water and photographed for documentation (Figure 4).

4.6. Data Statistics and Analysis

Data were analyzed in Excel. The transformation (positive root) rate (%) was calculated as: (number of explants producing positive transgenic hairy roots / number of explants producing hairy roots) × 100%.

5. Conclusions

This study demonstrates that *GRF*, *GIF* and *WOX* developmental regulators can enhance peanut hairy-root transformation and promote root growth. Compared with the empty-vector control, these treatments increased hairy-root induction and growth, raising the number of roots per explant by 1.3–2.4-fold and producing thicker, more highly branched roots. These tools provide a practical platform for peanut genetic engineering, including rapid gene function validation, CRISPR construct evaluation, and metabolite production.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: full-length coding sequences (CDSs) were amplified using gene-specific primers.

Author Contributions: X.L. supervised the study; X.L. and Y.C. designed the experiments; Q.Z., Y.C. and F.C. performed the experiments and analyzed the data; Q.Z. and Y.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data generated or analyzed during this study are included in this published article and its Supplementary Information Files.

Conflicts of Interest: The authors declare no conflicts of interest.

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Abbreviations

The following abbreviations are used in this manuscript:

GRF	Growth-Regulating Factor
GIF	GRF-Interacting Factor
WOX	WUSCHEL-related homeobox

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