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Systematic investigation and validation of peanut genetic transformation via the pollen tube injection method

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Abstract

Genetic transformation is a pivotal approach in plant genetic engineering. Peanut (*Arachis hypogaea* L.) is an important oil and cash crop, but the stable genetic transformation of peanut is still difficult and inefficient. Recently, the pollen tube injection pathway has been shown to be effective for the genetic transformation of peanut. However, the poor reproducibility of this pathway is still controversial. In this study, the appropriate time and location of injection, along with transgenic screening, were systematically investigated in the pollen tube mediated peanut genetic transformation. Our findings revealed that *Agrobacterium* injections could be conducted within a time window of two to three hours preceding and succeeding the blooming process. Among the various selective markers evaluated, the Basta screening emerged as the most expedient, followed closely by the DsRed visual screening. According to resistance screening and molecular identification, the average transformation efficiency was 2.6% in the heritable transgenic progenies, which was more likely affected by individual operation by style cavity injection. Furthermore, the use of synergistic FT artificially regulated the blooming of peanuts under indoor conditions, facilitating operations involving keel petal injection and ultimately enhancing the genetic transformation efficiency. Thus, our study systematically validated the feasibility of peanut genetic transformation through an optimized pollen-tube injection technique without tissue culture, potentially guiding future advancements in peanut engineering and molecular breeding programs.

Keywords Peanut, Pollen-tube injection, Genetic transformation, Transformation efficiency, Non-tissue culture transformation

Introduction

Genetic transformation of plants has powerful advantages for key gene function validation, not only by improving crop agricultural traits but also by accelerating the plant breeding process [1]. Depending on the delivery strategy, the introduction of foreign genes into plants can be achieved through diverse techniques, including *Agrobacterium* delivery, particle bombarding, viral infiltration, and naomaterial delivery. Among these methods, *Agrobacterium*-mediated genetic transformation is a widely utilized approach due to its simplicity and high efficiency. At present, both *Agrobacterium tumefaciens*

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and *Agrobacterium rhizogenes* were frequently used to mediate genetic transformation in various crops, such as rice, maize, wheat, and soybeans [2–4].

Until recently, the *Agrobacterium tumefaciens*-mediated floral dip method has been extensively applied in Arabidopsis genetic transformation since the previous study [5]. However, this method is not suitable for the majority of flowering plants, which possess limited quantities of flower buds and seeds. In contrast, *Agrobacterium rhizogenes*, which contain the *Ri* plasmid with the *rol* genes, facilitate the generation of abundant hairy roots and exhibit exceptional transformation efficiency in different plants [6, 7]. Recent studies have utilized the *Agrobacterium rhizogenes* derived hairy roots for gene editing and plant regeneration without tissue culture [8, 9]. Especially, the 'cut-dip-dudding' (CDB) method has emerged as a promising approach for genetic transformation and gene editing in several plant species that with root suckering capacity [8, 9]. Nevertheless, these transformation methods without tissue culture are largely not suitable for most plants whose regeneration process is difficult.

Peanut (*Arachis hypogaea* L.) is an important oil and food cash crop grown worldwide [10, 11]. To date, the genetic transformation for the cultivated peanut is difficult because of the complicated allotetraploid, lengthy regeneration process, and low proliferation coefficient in the tissue culture system. The scarcity of flower buds and seeds has further constrained the application of floral dip methods for peanut genetic transformation. Despite longstanding efforts in utilizing the *Agrobacterium rhizogenes* mediated hairy roots for genetic transformation of peanut [12, 13], achieving shoot regeneration from peanut hairy roots remains a significant hurdle. Additionally, the 'regenerative activity-dependent in plant injection delivery' (RAPID) method has been explored by using *Agrobacterium tumefaciens* [14], but its application often results in some chimeric transgenic progeny in peanuts [15]. Recent studies indicate that, only a handful of peanut species can be genetically transformed through tissue culture [16–21], or the pollen-tube pathway [22, 23], highlighting the need for further advancements in this field. Consequently, a robust genetic transformation system for peanut remains elusive.

The development of a stable, convenient, rapid and highly efficient genetic transformation method is urgently needed for peanut engineering. According to the recent studies, the pollen-tube injection pathway has emerged as a promising method for peanut genetic transformation, and has achieved to 73% of transformation efficiency [22, 24]. The pollen-tube injection method involves the transfer of DNA through the pollen tube channel to the nucellar and ultimately to the embryos. The specific procedures included pollen carrying, stigma dripping, ovary injection, and pollen injection [25]. The appropriate timing of the treatment allows foreign DNA to reach the fertilized but undivided egg cells [26, 27]. The concept of pollen injection was first reported by Zhou and colleagues [28], and it has been successfully use to create transgenic plants of several species, including *Solanum sisymbriifolium* [29–35], peanut [22, 24, 36], and peach [37]. However, pollen tube injection-mediated transformation is still controversial in peanuts because of poor repeatability. In this study, we comprehensively investigated and optimized the application of pollen-tube injection transformation in peanuts, by considering various factors including the injection operations, the transformed vectors, the selectable reporters, and progeny screenings.

Materials and methods

Plant material and growth conditions

As large as possible for pollen-tube injection, the seeds of the South China peanut variety *Zhanyou 75* were germinated and cultivated on an experimental farm (~ 150 m^2) in the biological park of South China Normal University (Supplementary Fig. S1a). To further manage the flowering process, peanut plants were cultivated in an artificial climate room under 16 h light / 8 h dark at 27 ± 2 ℃. The light period started at 9:00 a.m. and lasted until 1:00 a.m the next morning. The peanut plants grew well and produced more unblossomed flower buds at 7:00 ~ 8:00 a.m. for injection.

Vector construction

To investigate the efficiency of transformation screening, different selection markers, including the glufosinate (Basta), kanamycin (Kan), hygromycin (Hyg), and the stable red fluorescence protein (DsRed), were used in this study. Routine molecular cloning procedures were followed for plasmid construction as previously described [14, 38]. The vectors of pGreen (Kan), pCambia (Hyg), pCB302 (Basta), or pPTG (Kan) were used for overexpression, RNA interference (RNAi), or targeted mutation, respectively, with the corresponding selectable markers. The peanut genes of *AhUP1* (*Arachis hypogaea upward peg 1*, GeneBank: PQ083422), *AhDREB1* (*Arachis hypogaea dehydration responsive element binding protein 1*, GenBank: KU143745.1), and *AhPDK1* (*Arachis hypogaea pyruvate dehydrogenase kinase 1*, PeanutBase: *Arahy.I3NY02*) were selected as examples for different delivery targets. Among these genes, *AhDREB1* is the one we have previously reported before [39], while the others are the related target genes currently under investigation by our group. Briefly, the open reading frames (ORFs) of *AhUP1* and *AhPDK1* were amplified and inserted into the pCambia vector under the control of the *Arachis hypogaea ubiquitin 10* (UBQ10) promoter [15] to generate

mCherry-*AhUP1* and mCherry-*AhPDK1*, respectively, while the ORF of *AhDREB1* and *AhPDK1* were amplified and cloned into the pGreen vector [40] under the control of the *cauliflower mosaic virus 35 S* (35 S) promoter to generate *AhPDK1*-GFP and *AhDREB1*-GFP, respectively. The synthetic guide RNA (sgRNA) sequences for the *AhUP1* and *AhDREB1* were designed and inserted into the pPTG vector [41] under control of the the *Arabidopsis U6-26* (*U6*) promoter and between the original tRNA and scaffold to construct the *AhUP1*-Cas9 and *AhDREB1*-Cas9, respectively. The corresponding artificial microRNA (amiRNA) products for *AhUP1* and *AhDREB1* were inserted into the HBT vector and then cloned into the pCB302 plasmid [42] to generate the amiR-*AhUP1* and amiR-*AhDREB1*, respectively. Moreover, the ORF of *glucuronidase* (*GUS*) was used to replace the original amiRNA frame in the DsRed-assisted vector [43] for GUS report application in peanut. In this system, the expressed DsRed protein facilitated the screening in peanuts by visualizing the transgenic seeds with red fluorescence, and the expressed *FLOWERING LOCUS T* (*FT*) promoted more blooming for pollen-tube injection. The constructed plasmids were subsequently transformed into *Agrobacterium tumefaciens* strains (AGL1 or GV3101). All *Agrobacterium* clones were further confirmed via PCR sequencing before injection. Some of them have also been transformed into peanut somatic embryos via tissue culture for additional verification.

Pollen-tube injection transformation

During the flowering period, the *Agrobacterium tumefaciens* mediated pollen-tube pathway was performed in peanut as described previously [44] and properly modified in our investigation. Fresh bacterial suspensions were prepared, and 100 μM acetosyringone (AS), 10 mM methane sulfonic acid (MES), and 10 mM $MgCl_2{\cdot}6H_2O$ were added to the suspensions. All flowers and gynophores were removed from the recipient plants before infection. Then, the suspension (~ 0.1 mL) was injected into the style cavity or keel petal using the injector needles. Each peanut plant received continuous injections for a period of 15 days, and each flower was injected. In each independent experiment, at least 20 peanut plants were injected, or even more. Finally, the unmarked flowers were removed and until the ripe pods were harvested for further screening and identification.

Resistance screening of the transgenic peanut progeny

The pods labeled with the injected marker were harvested and dried out after maturation. These seeds were then surface sterilized. The seeds were immersed in 75% EtOH for 1 min, and then throwing up the EtOH, and immediately following 10% (v/v) sodium hypochlorite was added for 10–15 min, and the seeds were subsequently rinsed 6 times with sterile distilled water. Finally, the seeds were immersed into sterile water overnight. The next day, the seeds were soaked in sterile napkins and planted on the MS (Murashige and Skoog) selective medium supplemented with different antibiotics. For Basta screening, peanut seeds were immersed in distilled water overnight, and then planted into the soil until the four-leaf stage, and a solution containing 0.01% (v/v) Basta was sprayed onto the leaves.

PCR detection of the transgenic peanut progeny

For genomic DNA detection, the leaf tissues from those seedlings after resistance screening were ground for DNA extracting using cetyltrimethylammonium bromide (CTAB) method as previously described [45]. Routine PCR procedure was performed in a total volume of 25 μL with specific primers (Supplementary Table S1). The PCR products were detected by electrophoresis and further sequencing. The product sequences were analyzed and aligned via SnapGene®6.0 software. For gene expression detection, total RNA was extracted via the Total RNA Extraction Kit (Promega, LS1040, USA) and reverse-transcribed via the M-MLV reverse transcriptase (Promega, M1705, USA). Quantitative reverse transcription PCR (qPCR) was performed in triplicate on a Roche LightCycler 480 real-time system with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711, China) following the manufacturer's instructions. The relative expression levels of the target genes were normalized to the internal control *Arachis hypogaea Actin* (GenBank: DQ873525.1) as previously described [46]. The primers used are listed in Supplementary Table S1.

Immunoblot detection of the transgenic peanut progeny

To analyse the protein expression levels of the target genes, the leaf tissues or seeds were harvested and total protein was extracted according to the protocol of the Plant Protein Extraction Kit (Solarbio, BC3720, China). Finally, the protein samples were subjected to 10% SDS-PAGE, immunoblotted with anti-mCherrry (Biodragon, B1153, China), and stained with Coomassie blue solution (Beyotime, P0017, China).

Results

Time optimization for pollen tube injection transformation in peanuts

To determine the appropriate time for pollen tube injection in peanuts, the anther dehiscence and germination, as well as pollination in peanuts were carefully monitored over a time course. On sunny days, the anthers dehisced and were released before 6:00 a.m. Although the flowers still did not bloom, they gradually germinated until 8:00 a.m. (Fig. 1a, b). All of those pollen grains germinated on the stigma between 9:00 a.m. and 10:00 a.m.

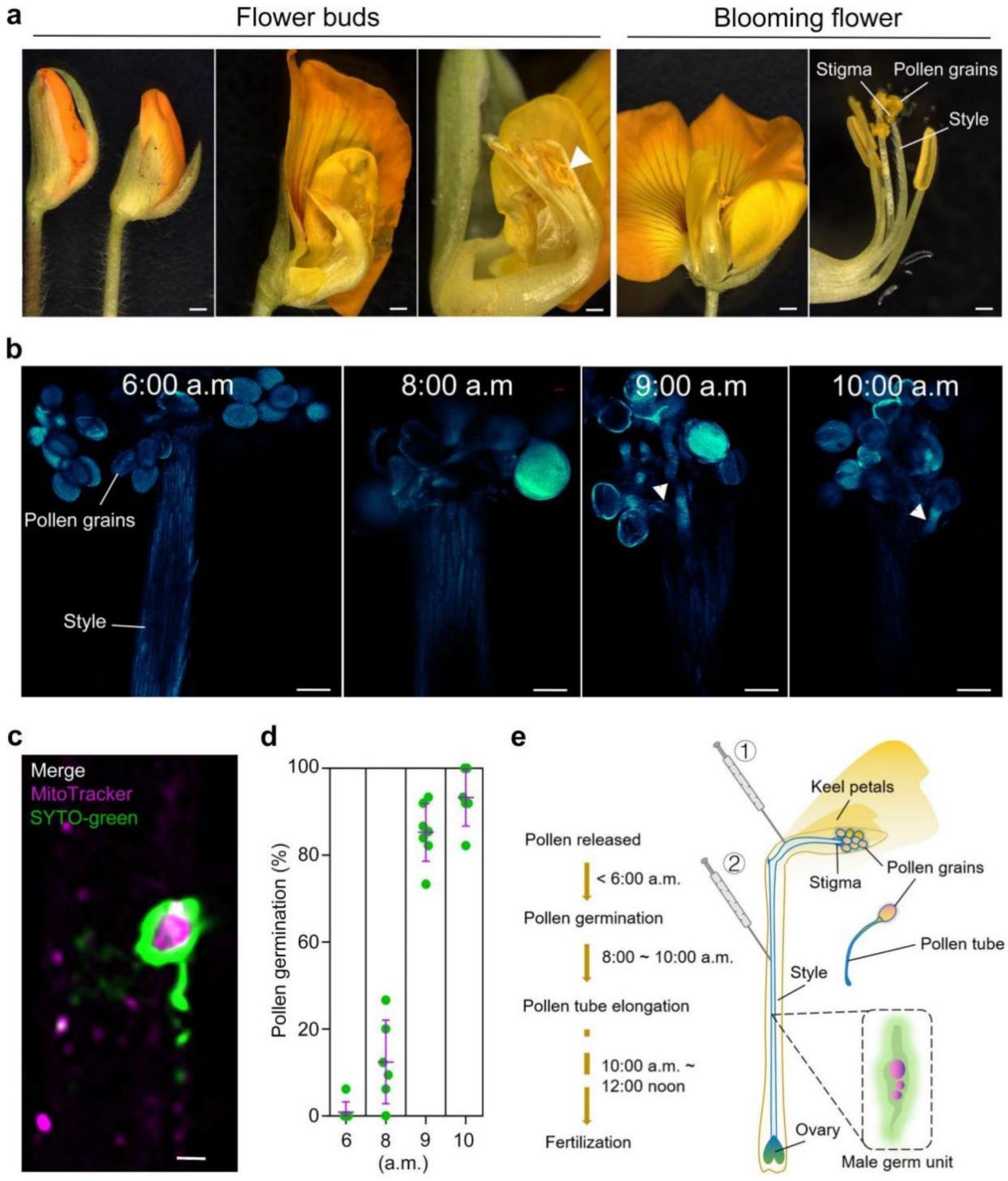

Fig. 1 Illustration of pollen-tube growth and injection time window in peanuts. **a**, Depiction of the flower bud and the blooming flower of peanut. Scale bars: 0.5 cm. **b**, A time-lapse study was conducted to observe the germination and elongation of pollen grains on the stigma of peanut plants. The nascent pollen tube is denoted by a white triangle. Scale bars represent 20 μm. **c**, The 'male germ unit' was observed by merging SYTO™ (fluorescent dye for nucleotides, green) and MitoTracker™ (fluorescent dye for mitochondria, pink purple) under a confocal microscope. Scale bars, 2 μm. **d**, Germination rates of pollen grains at different times during the morning. **e**, Diagram illustrating the process of transformation in peanut via two pollen tube injection strategies on sunny days. ① indicates that *Agrobacterium* is injected into the keel petals prior to blooming; ② indicates that *Agrobacterium* is directly injected into the style cavity during the optimal time window

(Fig. 1d). The 'male germ unit' looked like a water droplet that elongated along the style filament and ultimately reached the ovary (Fig. 1c; Supplementary Fig. S2), which was typically viewed as a sign of pollen germination and tube elongation [47, 48]. The stigma of peanuts is directly connected to the ovary via a long style filament, which is approximately 2 ~ 6 cm in length, or even more longer (Fig. S2c), suggesting that the results of injecting into keel petals and styles may be the same if these pollen grains have germinated.

The 'male germ unit' could be observed within the style cavity or on its wall from 10:00 a.m. to 12:00 noon (Fig. 1c). On the basis of these observations, the window for pollen tube injection appears to be relatively broad, extending from approximately 6:00 a.m to 12:00 noon under the sunny conditions. This window may be further extended in cloudy weather. To maximize the efficiency of the pollen tube injection transformation, two injection strategies were employed as shown in Fig. 1e. The one strategy is that *Agrobacterium* are injected into the keel petals prior to blooming, and the another strategy is that they are directly injected into the style cavity during the optimal time window from 9:00 a.m. to 10:00 a.m. Notably, those *Agrobacterium* are introduced into the style cavity rather than the pollen tube, which serves as the passageway for the pollen tube to traverse.

Optimization resistance screening in seeds obtained via style cavity injection method

Appropriate Kan resistance screening

Efficient and rapid screening techniques play a crucial role in the genetic transformation of peanuts via style cavity injection method. In a previous study, we found that peanut embryos and seeds exhibit varying degrees of resistance to kanamycin and hygromycin. Peanut somatic embryos are quite sensitive to kanamycin, making them suitable for somatic embryo screening during tissue culture. Kanamycin (Kan) is generally utilized at a concentration of 50 mg/L for screening the resistant seedlings of various species. However, most peanut seeds germinated and thrived at this concentration. Notably, 33.9% of the *AhPDK1*-Cas9 seedlings presented apparent Kan resistance at 50 mg/L, but the further PCR analysis revealed that no transgene insertion was detected among these resistant seedlings (Table 1), suggesting that this concentration is not inadequate for transformation screening in peanuts. Subsequently, the effective concentration of Kan required for resistance screening was determined to be at least 100 mg/L in our study (Table 1). At this concentration, a total of thirty-nine *AhDREB1*-Cas9 and thirteen *AhUP1*-Cas9 Kan-resistant seedlings were successfully obtained, respectively (Fig. 2b; Table 1). These selected seedlings were further validated via PCR identification (Fig. S3; Table 1). Among them, most of the *AhUP1*-Cas9 transgenic lines were positive according to PCR via the

Table 1 Transformation efficiency of style cavity injection method

Groups	Vectors	Antibiotics (mg/L, m/v)	Total seeds	Resistant seedlings	Screening rate (%)	*Molecular identification	Positive seedlings	Positive rate (%)	**Transformation efficiency (%)
Experiment A	*AhDREB1-GFP*	Kan (100)	380	46	9.5	36	12	33.3	3.2
	amiR-AhDREB1	Bar (0.1%)	480	39	7.1	34	21	61.8	4.4
	AhDREB1-Cas9	Kan (100)	520	49	7.5	39	15	38.5	2.9
Experiment B	*AhUP1-Cas9*	Kan (100)	348	13	3.7	13	13	100	3.7
	AhUP1-Cas9	Kan (50)	124	42	33.9	42	0	0	0
Experiment C	*mCherry-AhPDK1*	Hyg (50)	148	16	10.1	15	3	2.0	0.2
	AhPDK1-GFP	Kan (50)	235	47	20	47	0	0	0
Experiment D	*mCherry-AhUP1*	Hyg (50)	494	38	7.7	36	22	61.1	4.7
	amiR-AhUP1	Bar (0.1%)	786	103	13.1	80	26	32.5	4.3

* Number of resistant seedlings selected for molecular identification.

** The transformation efficiency was calculated by multiplying the positive value of resistant screening (%) with the positive value of molecular identification (%).

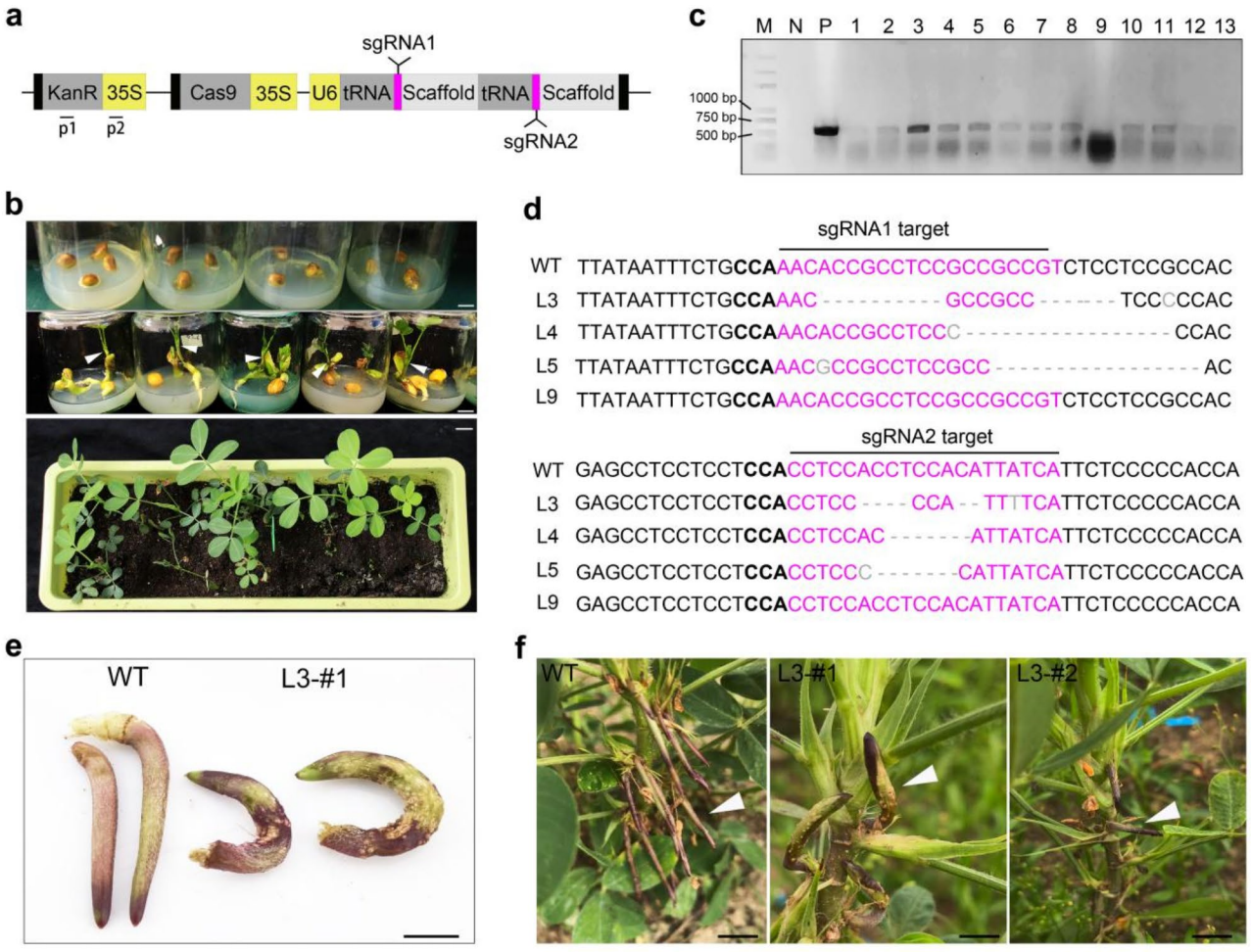

Fig. 2 Kanamycin screening and identification for gene editing application via pollen-tube transformation in peanut. **a**, Schematic representation of the *AhUP1*-Cas9 cassette. KanR, kanamycin (Kan) resistance gene; 35S, *cauliflower mosaic virus 35S* promoter; U6, *Arabidopsis U6-26* promoter; sgRNA1/2, two guide RNAs for target gene editing. **b**, The Kan-resistant seedlings were screened with the selective medium (upper) and subsequently transplanted into soil for further cultivation (bottom). Scale bars: 1 cm. **c**, The Kan-resistant seedlings were identified by PCR using the specific primers. M, DNA marker; N, water was used as the negative control; P, the plasmid was used as the positive control; 1 ~ 13, represent the thirteen Kan-resistant samples. The primers of p1 and p2 were designed to amplify the specific fragment of T-DNA. **d**, DNA sequencing for target gene mutation. L1 ~ 13: the transgenic lines. **e**, Phenotypes of peanut pegs from the WT and transgenic lines. Scale bar: 1 cm. **f**, Phenotype of the WT and transgenic lines in the field. Scale bars: 1 cm

primes of the target sequence (Fig. 2a, c). Additionally, three representative positive lines (L3, L4, and L5) represented the homozygous *AhUP1* gene mutations in the sgRNA targeted regions by sequencing and alignment analysis (Fig. 2d; Fig. S3). Notably, although some plants initially showed weak Kan resistance on the medium, such as L9, which ultimately perished after transplantation into the soil for a prolonged period, mostly due to false positives (Fig. 2c, d). Correspondingly, the false positive sample of L9 had no sequence change in the sgRNA targeted regions (Fig. 2d). Besides, the growth direction of the pegs were upward in the *AhUP1* mutant line 3 (L3), indicating its geotropism was lost, comparing with the normal geotropic pegs from the WT plants (Fig. 2e, f), which further supports the achievable gene-editing application in peanut via pollen-tube injection transformation. These findings underscore the feasibility of establishing transgenic peanuts via a style cavity injection strategy and highlight the importance of the appropriate concentration for enhancing effective positive screening.

Resistance screening of Hyg and Basta decreased the frequency of false positives

To further evaluate the effectiveness of the style cavity injection-based transformation screening, the transgenic seeds constitutively expressing mCherry-*AhUP1* or mCherry-*AhPDK1* were individually subjected to screening for hygromycin resistance, and the results revealed that three mCherry-*PDK1* and twenty-two mCherry-*AhUP1* transgenic plants were successfully identified by hygromycin resistance and molecular analysis (Table 1;

Fig. 3a-c). Therein, the mRNA expression of *AhUP1* was detected by qPCR and was significantly increased in the mCherry-*AhUP1* transgenic lines (Fig. 3d). Correspondingly, the higher expression of the mCherry fusion protein was detected in the related lines, which showed the higher transcript levels (Fig. 3e). Notably, peanut seeds were relatively sensitive to hygromycin (50 mg/L), because they were severely hampered except in the case of the transgenic plants (Fig. 3b). However, both hygromycin and kanamycin resistance screenings were conducted on the resistant selectable medium with rigorous aseptic manipulation, and a substantial number of sterile containers were used for these screening thereby posing inconveniences.

Therefore, the non-sterile Basta resistance screening was conducted on the transgenic plants generated through style cavity injection. The amiRNA-*AhDREB1* seeds were subjected to imbibition and subsequently cultivated directly in soil. Then, a 0.01% Basta was applied and sprayed onto the peanut leaves at the four-leaf stage. Five days later, a majority of the leaves presented signs of wilting and mortality, whereas certain plants continued to produce a limited number of fresh leaves (Fig. 4b). To eliminate the possibility of false positives, each plant was sprayed three times with a five-day interval between each application. Leaves from the seedlings that remained fully green were collected for PCR detection by using specific primers of the genes encoding *Bar* or *GFP* (Fig. 4c). The results of the expression and phenotypical analyses revealed *AhDREB1* knockdown and curled leaves in the transgenic lines compared with those in the WT, indicating that the amiRNA-*AhDREB1* cassette was transformed and functioned (Fig. 4d-f). Ultimately, twenty-one amiRNA-*AhDREB1* resistant plants were successfully identified with the good efficiency by resistance to Basta (Table 1).

Collectively, these findings further support the feasibility of obtaining transgenic progeny by the style cavity

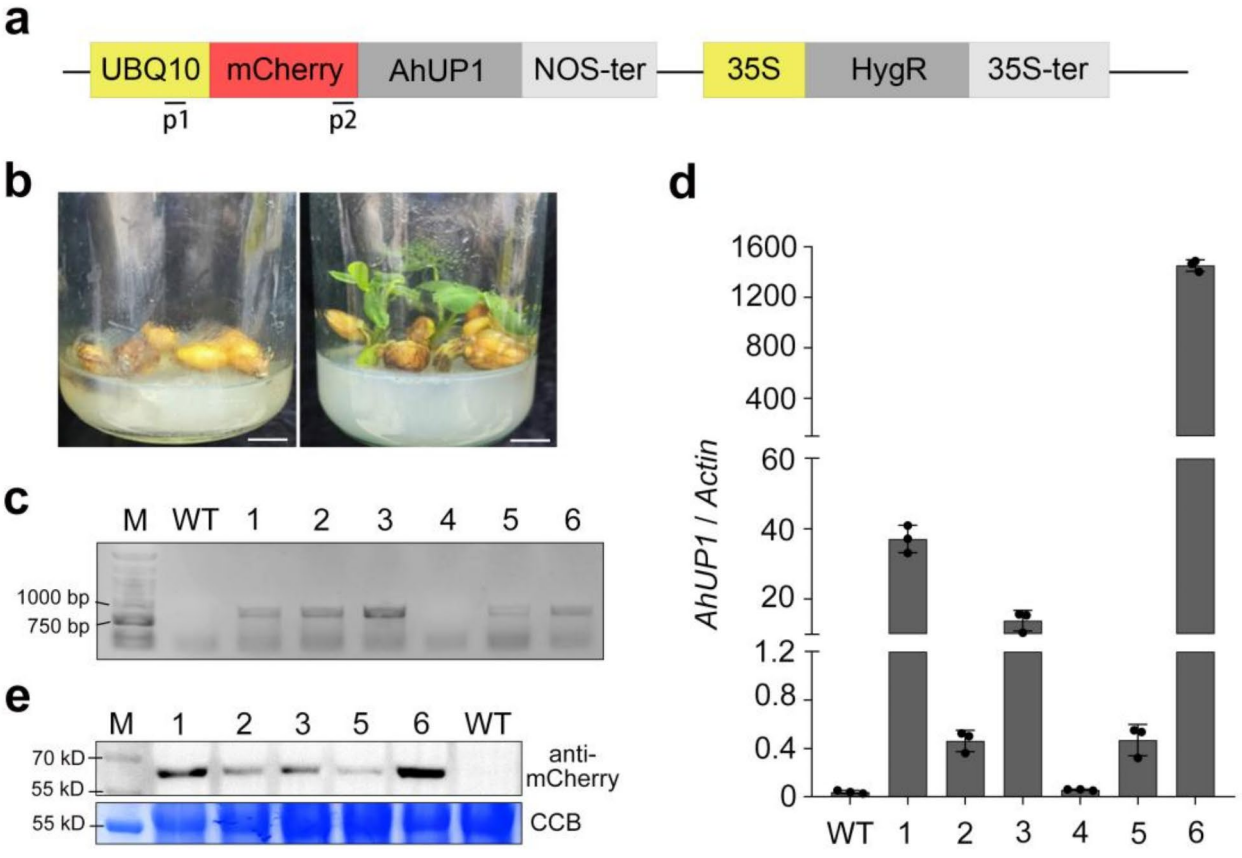

Fig. 3 Screening and identification of the overexpressing transgenic peanuts. **a**, Schematic overview of the mCherry-*AhUP1* overexpression cassette. UBQ10, *Arachis hypogaea ubiquitin 10* promoter; 35S, *Caulifower mosaic virus 35S* promoter; 35S-ter, *35S* terminator. **b**, The *AhUP1* transgenic plants were screened on the medium supplemented with hygromycin (Hyg, 50 mg/L). Scale bars,1 cm. **c**, PCR detection of mCherry-*AhUP1* in the Hyg-resistant transgenic lines. Genomic DNA was extracted from the leaves of the wild type (WT) and the transgenic lines (1 ~ 6), and PCR was performed to amplify a specific fragment of the *mCherry-AhUP1* cassette. M, DNA marker. **d**, qPCR detection of *AhUP1* mRNA expression in the WT and transgenic lines. **e**, Western blot detection of the mCherry-AhUP1 protein expression in the WT and different transgenic lines. M, protein marker; CCB, Coomassie brilliant blue staining as the loading control

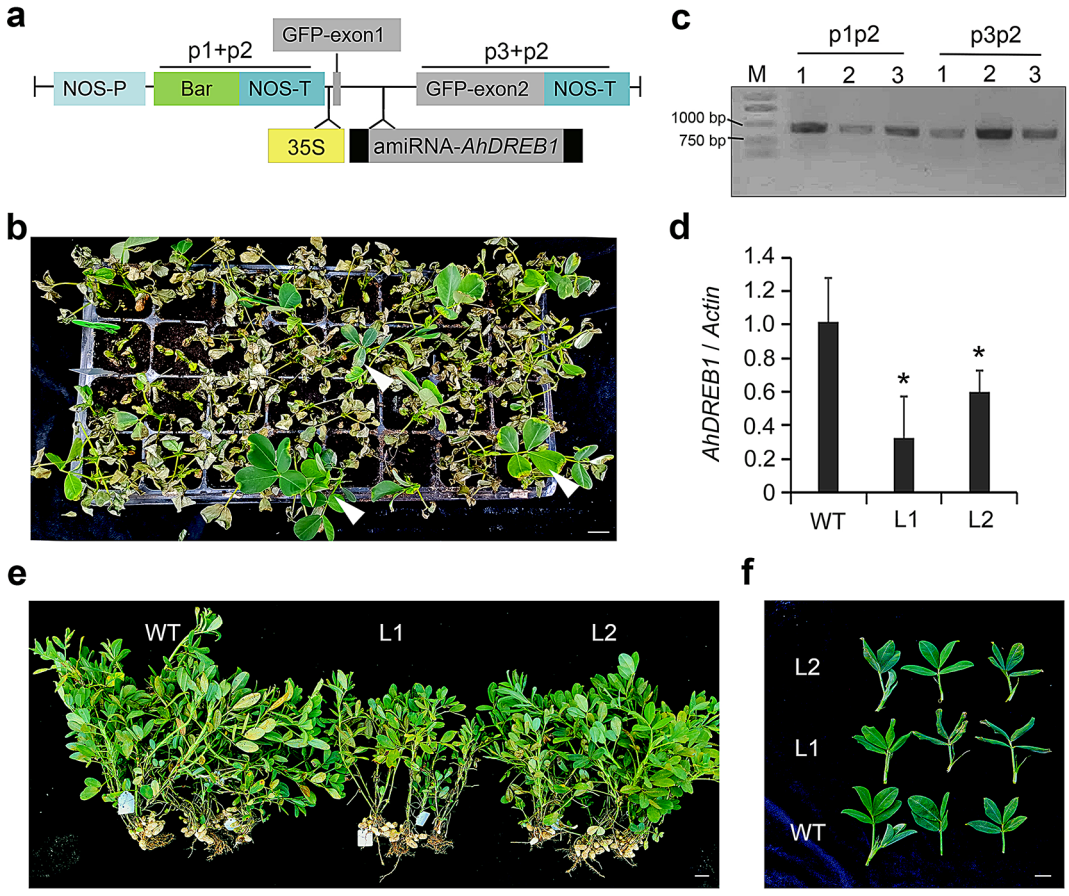

Fig. 4 Screening and identification of the amiRNA-*AhDREB1* transgenic peanuts. **a**, Schematic overview of the amiRNA-*AhDREB1* cassette. NOS-P, *nicotinamide synthetase* promoter; NOS-T, *nicotinamide synthetase* terminator; black boxes indicate intron borders; **b**, Transgenic peanut seedlings were screened by spraying 0.01% Basta. The asterisk denotes the seedlings that exhibited complete greenness without any signs of leaf withering after undergoing three consecutive treatments with Basta. Scale bar represents 1 cm. **c**, PCR amplification was performed to detect the presence of the *Bar* and *GFP* in the amiRNA-*AhDREB1* transgenic lines. M, DNA marker; the primers of p1p2 and p3p2 were designed to specifically amplify two specific fragments as shown in **a**. **d**, qPCR detection for *AhDREB1* mRNA expression in the wild type (WT) and transgenic lines (L1 and L2). Asterisks (*) indicate significant differences compared with the wild type ($P < 0.05$). **e**, Phenotype of 12-week-old peanut plants from the WT the transgenic lines in the experimental field. Scale bar: 1 cm. **f**, Phenotype of leaves from the WT and the transgenic lines. Scale bars: 1 cm

injection method in peanuts. In addition, the practical convenience of Basta in screening resistant transgenic peanuts was highlighted.

Transformation efficiency of the style cavity injection method

As mentioned above, nine vectors carrying diverse marker genes were introduced into peanut by *Agrobacterium*-mediated style cavity injection in the individual experiments (Table 1). Most of them were effectively transformed, and the positive progenies could be obtained and confirmed through a combination of resistance screening and molecular identification. Overall, the average transformation efficiency was 2.6% (Table 1). Apparently, the individual operation had a significant impact on this efficiency, as the rate rose to 3.8% upon excluding experimental group C from the analysis. All the progenies of these genetically identical plants were subsequently transplanted into soil for further evaluation of the stability of the transferred genes and their functions. However, the transformation efficiency is far bellow when compared to that reported in a previously published study [44].

The DsRed-FT-assisted visual system promoted transformation efficiency via keel petal injection

Given the lower transformation efficiency obtained by the style cavity injection method in our experiments than in a previous published report [44], we decided to reiterate the injection site on the keel petals as the second strategy to address this (Fig. 1e), and several modifications were simultaneously carried out according to our observations. First, the DsRed-FT-assisted system was modified and used for the purpose of the visual marker screening in peanut (Fig. 5a). The constitutively expressed DsRed marker provided a convenient means for selecting

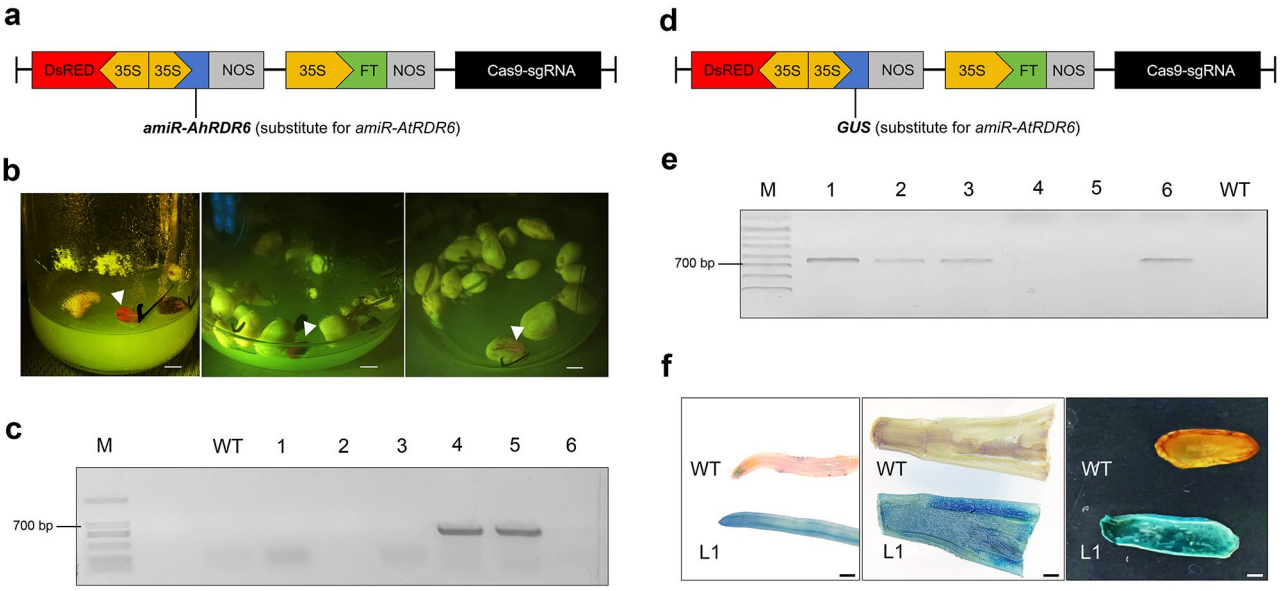

Fig. 5 Transformation of the DsRed-FT-assisted visual system into peanut and the GUS report application by the keel petal injection strategy. **a**, Schematic overview of the T-DNA region within the modified DsRed-FT vector. 35S, *cauliflower mosaic virus 35S* promoter; FT, coding region of the *FLOWERING LOCUS T* from Arabidopsis (AT1G65480); NOS, NOS terminator; Cas9-sgRNA, the original CRISPR/Cas9 elements; DsRed, red fluorescent protein; amiR-AhRDR6, artificial miRNA targeting peanut *RNA-dependent RNA polymerases 6* (*AhRDR6*) replace Arabidopsis *amiR-AtRDR6*. **b**, Fluorescence of T1 generation transgenic peanut seeds. The DsRed-positive seeds were indicated by white triangles. Scale bars: 1 cm. **c**, the T-DNA sequences were detected by PCR for the DsRed-positive lines. M, DL2000 marker. WT, wild type; 1 ~ 3, genomic DNA from the harvested seeds without fluorescence. 4–6, Genomic DNA from the harvested seeds with fluorescence. **d**, Schematic overview of the *GUS* in the DsRed-FT vector. **e**, the T-DNA sequences were detected by PCR for the positive lines. M, DL2000 marker; WT, wild type; 1 ~ 6, genomic DNA from the transgenic positive lines with DsRed fluorescence. **f**, GUS histochemical staining of the roots, stem, and shell from the WT and transgenic positive line. Scale bars: 0.5 cm

transgenic seeds, and an amiRNA targeting *Arachis hypogaea RNA-dependent RNA polymerase 6* (amiR-*AhRDR6*) was used to replace the original amiR-*AtRDR6* within the system with the expectation that it would enhance the compatibility of transformation in peanut (Fig. 5a). Second, to facilitate the repeated keel petal injection and operational convenience, the flowering process was properly delayed in peanuts by indoor light control. Specifically, the two-week-old peanut plants were planted and maintained in a growth room under a controllable photoperiod of long-day conditions (Fig S1b). The transformation operation was then conducted between 8 a.m. and 10 a.m., and most budding flowers were observed during this period due to the continuous *FT* expression (Fig S1e). Under these conditions, each peanut plant conveniently underwent continuous injection for a period of 15 days, with each budding flower being injected on the keel petals. Ultimately, a total of 75 seeds were harvested from these plants, 5 of which exhibited apparent red fluorescence according to visual screening by using a fluorescent flashlight (Fig. 5b). These findings indicated that the transformation efficiency (5/75, 6.6%) of keel petal injection was higher than that of style cavity injection (average 2.6%; Table 1), despite minor improvements compared with the reported previous study. Notably, among the five seeds, only two seeds germinated and could be detected by target PCR amplification (Fig. 5c), suggesting that suppressing the expression of *AhRDR6* may affect seed germination in peanut. Therefore, the transformation of the *GUS* reporter gene was conducted in peanut by replacing the amiRNA-*AhRDR6* (Fig. 5d). The results revealed normal seed germination and a relatively higher positive rate (Fig. 5e), and the GUS staining was observed in the positive roots, stems, and pods (Fig. 5f), further validating these transgenic events through the keel petal injection approach.

Discussion

In summary, we investigated transformation of peanuts by the optimized pollen tube injections, either at keel petals or into the style cavity. The appropriate time window for injection is determined to be two to three hours before and after pollination, which can be conveniently managed in an artificial climate chamber to delay the flowering process and provide sufficient time for ease of operation. Collectively, our findings suggest that Basta resistance serves as the most convenient approach for peanut progeny screening, given the large size of peanut seeds and the requirement for numerous sterile containers for cultivation through other resistance screening methods. At the natural experimental farm, the average transformation efficiency ranged from 2.6 to 3.8%,

which was influenced mainly by the practical executor. A minor increase in transformation efficiency is observed when the flowering process is managed through keel petal injections. However, the transformation efficiency remains significantly lower than that reported in previous study. Our study aims to clarify the feasibility of the transformation method via the optimized pollen-tube injection method in peanut.

The genetic transformation technique has widely and successfully improved the yield and quality of numerous major crop species, including soybean, rice, maize, and cotton. Nevertheless, compared with that in other crops, the genetic transformation technique in peanut remains relatively rare, and positive screening is often inefficient. Rapid production of the heritable transgenic plants relies mainly on the efficient transformation methods, while appropriate screening markers will facilitate the selection of transgenic generations. The size of peanut seeds and the abundance of endophytes pose significant challenges in seed disinfection and cultivation processes. This is highly inconvenient when screening large quantities of peanut seeds via kanamycin or hygromycin resistance. DsRed-labelling has widely applied to identify the transgenic seeds by visual screening in plants due to its superior sensitivity compared with GFP [49]. In our investigations, the utilization of Basta resistance and DsRed-labelling marker has emerged as a viable alternative for screening transgenic peanuts (Fig. 4). Additionally, introducing the amiR-*AhRDR6* into the DsRed-FT-assisted system is anticipated to potentially enhance gene-editing as previously observed in *Arabidopsis* [43], and it is expected to promote more flowering in peanuts for pollen tube injection. However, our studies indicated that the down-regulation of *AhRDR6* expression may potentially compromise the germination and growth of peanuts, resulting in the inability of certain seeds exhibiting red fluorescence to survive (Fig. 5b). In fact, fertility and silique development are also impaired in *Arabidopsis* mutants lacking both the *RDR1* and *RDR6* genes [50]. These findings underscore the necessity for further refinement of this system. Notably, the injection of the DsRed-FT-assisted system has significantly increased the floral production in T0 peanut plants, thereby providing a more abundant recipient of materials for pollen-tube injection. This promising system offers potential advancements for enhancing the efficiency of transformation in peanuts.

On the basis of a previously published report by Zhou and colleagues [44], by using the style cavity injection method, our transformation efficiency falls below expectations, reaching a mere 2.9–4.3%, which is significantly lags behind the reported efficiency of 50% ~ 73.3%. To address this problem, many measures have been taken to ensure that peanut grow well and produce sufficient blooms and fruits, such as deliberately delaying the flowering process, maintaining a suitable temperature and providing adequate nutrients. In our study, by using the keel petal injection method, *Agrobacterium tumefaciens* mediated peanut transformation was improved by controlling the light conditions and optimizing the delivery strategy through proper screening. Finally, only 6.6% positive plants were obtained, which is still far below previously reported percentage. Nevertheless, this efficiency is close to the transformation frequency of *Agrobacterium* immersion or particle bombardment under peanut tissue culture. Previously, a relatively stable somatic embryo induction and transformation method was established in our laboratory (Supplementary Fig. S4). When *Agrobacterium tumefaciens* was used to immerse the somatic embryos of peanuts, the transformation frequency ranged from 3 to 5%, while the transformation rate of somatic embryos by particle bombardment reached up to 10%. Compared with the pollen-tube injection method, the genetic transformation of somatic embryos offers greater stability and reliability, albeit with a longer procedure. Specifically, the successful regeneration of seedlings requires approximately nine months via somatic embryo transformation by *Agrobacterium* immersion, but at this time, the identification of the T1 generation positive seedlings has been completed by pollen tube injection transformation and transplantation into the soil.

In conclusion, we have investigated the genetic transformation of peanuts by the systematic optimization of pollen-tube injection methods. Although our findings confirm the validity of this method, the transformation efficiency remains significantly lower than expected. Thus, several unresolved questions remain. First, the accuracy of screening may vary between our study and others. In our case, we confirmed the positive plants through both resistance screening and molecular identification, which effectively eliminated the numerous false positives and the unstable or unheritable transgenic events. Additionally, the pollen tube injection method offers the advantage of a shorter procedure than does the somatic embryo transformation system in peanuts. Properly delaying the flowering process under controlled conditions and selecting appropriate screening methods could effectively enhance the operational feasibility of the keel petal injection approach.

Supplementary Information

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Supplementary Material 1

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Author contributions
Xiaoyun Li and Xu Liu conceived and designed the overall research. Chen Huang and Xiaomeng Li performed plasmid construction. Chen Huang, Chen Yang, Huifang Yang, Yadi Gong, and Lexin Li performed plant transformation and screening identification. Chen Huang and Chen Yang drafted the manuscript. All the authors contributed to the data analysis and manuscript preparation.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agree with the submission of this manuscript to Plant Methods.

Competing interests
The authors declare no competing interests.

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