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REVIEW

Current status of the genetic transformation of *Arachis* plants

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Highlights

- Functional validation of key peanut genes is impeded by the lack of an efficient genetic transformation system.
- Visible phenotypic markers serve as ideal tools for confirming transgenic events in peanut.

Abstract

Peanuts (*Arachis hypogaea*) are important sources of vegetable oil, protein, and forage. The genus *Arachis* comprises nine intrageneric taxonomic sections encompassing 84 species. Most *Arachis* species are wild plants that serve widely as forage and turfgrass. Furthermore, wild *Arachis* species provide valuable gene resources for broadening the genetic diversity of cultivated peanuts. To date, several key genes have been identified through the use of recombinant inbred lines derived from interspecific crosses within *Arachis*. Despite this progress, the application of genetic engineering to enhance peanut traits remains limited. This limitation arises primarily from the absence of a robust and reliable genetic transformation protocol for *Arachis* species. Nevertheless, evidence indicates that successful genetic transformation of *Arachis* plants was first reported approximately 30 years ago. Thus, a notable discrepancy exists between early reports of transformation success and the ongoing challenges in stably transferring candidate genes into *Arachis* genotypes. This review summarizes existing methods for regeneration and genetic transformation in *Arachis*, aiming to advance understanding of transgenic technologies applicable to this genus.

Keywords: *Arachis*, CRISPR/Cas9, genetic transformation, *in vitro* regeneration

1. Introduction

Peanut (*Arachis hypogaea*) originated in South America through hybridization between *A. duranensis* and *A. ipaensis*, followed by chromosomal doubling, making it an allotetraploid (Bertioli *et al.* 2016, 2019). Peanuts are a source of oil and protein for human consumption and serve as forage for livestock (Song *et al.* 2023). The genomes of cultivated peanut and its progenitors have been fully sequenced, accelerating the identification of key genes associated with various phenotypes (Bertioli *et al.* 2016, 2019; Yin *et al.* 2018; Chen *et al.* 2019; Zhuang *et al.* 2019). Moreover, numerous recombinant inbred lines (RILs) have been developed to identify key gene markers in peanuts. Genes regulating testa color and pod size have been identified using RIL populations, and their functions validated in *Arabidopsis thaliana* and *Nicotiana tabacum via Agrobacterium*-mediated transformation (Zhao *et al.* 2020, 2023; Yang *et al.* 2023). However, the functional roles of these genes in *Arachis* species remain unconfirmed, primarily due to the absence of an efficient genetic transformation protocol. *In vitro* regeneration and genetic transformation studies in *Arachis* were first reported three decades ago. Numerous transgenic *Arachis* lines have since been generated, harboring genes conferring abiotic and biotic stress tolerance, among other traits (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021). However, the discrepancy between genetic transformation and functional validation of unknown genes in *Arachis* stems from low transformation efficiency and insufficiently optimized genetic engineering approaches. This review summarizes advances in *in vitro* regeneration and genetic transformation of *Arachis* species.

2. *Arachis* plant regeneration

Since the first *in vitro* regeneration protocol for *Arachis* species was published in the 1990s, both *in planta* and tissue culture-based regeneration systems have been established. *In planta* regeneration relies on direct differentiation of

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explants through new developmental pathways, whereas tissue culture regeneration involves dedifferentiation into callus followed by redifferentiation into whole plants. Tissue culture regeneration is highly genotypic dependent and requires callus induction on media such as Murashige and Skoog (MS), while *in planta* regeneration is genotype-independent. In *Arachis*, *in planta* regeneration achieves higher efficiency than tissue culture methods. Therefore, *in planta* regeneration is the preferred method for *in vitro* propagation of *Arachis* plants.

2.1. *In planta* regeneration

In vitro regeneration of *Arachis* species can utilize cotyledons, cotyledonary nodes, de-embryonated cotyledons, embryonic axes, leaflets, hypocotyls, epicotyls, axillary buds, immature leaves, or zygotic embryos as explants. Among these, cotyledons and cotyledonary nodes exhibit the highest regeneration rates (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021). *In planta* regeneration is genotype-independent and does not require explant dedifferentiation. The application of appropriate phytohormones to induce shoot and root formation is the most critical factor. For instance, combining thidiazuron with proline enhances somatic embryogenesis and organogenesis (Murch *et al.* 1999; Joshi *et al.* 2003). A mixture of 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) proves more effective than BAP with 2,4-dichlorophenoxyacetic acid (2,4-D) for root induction from cotyledonary nodes during *in vitro* regeneration (Lamboro *et al.* 2022).

In vitro regeneration of cultivated peanut has been well documented (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021). This review also compiles data on wild peanut species. Immature zygotic embryos, quartered seeds, and cotyledonary nodes have enabled somatic embryogenesis and regeneration in *A. paraguariensis* (Sellars *et al.* 1990; Aina *et al.* 2012a, b). In *A. archeri* and *A. appressipila*, seed treatment with BAP induces somatic embryogenesis (Pacheco *et al.* 2007). Similarly, embryonic axes treated with BAP regenerate *A. stenosperma* plants (Pacheco *et al.* 2008). For *A. glabrata*, cotyledonary nodes cultured on MS medium supplemented with 5 mg L^{-1} of meta-topolin produce shoots, while 2 mg L^{-1} NAA and 0.2% activated charcoal promote root formation *in vitro* (Pholjad *et al.* 2020). Cotyledonary nodes of *A. diogoi*, *A. stenosperma*, *A. duranensis*, *A. cardenasii*, and *A. correntina* have also been successfully used for *in vitro* regeneration (Srinivasan *et al.* 2010).

Recent studies report that supplementary *Agrobacterium rhizogenes* can induce hairy roots in *Arachis* species. Seeds and leaves serve as effective explants for hairy root induction (Fig. 1), offering a simple approach to facilitate genetic transformation (see below under *Hairy root transformation*). Seeds of *A. paraguariensis* and leaves of *A. duranensis* and *A. ipaensis*, all wild *Arachis* species, have been used to generate hairy roots (Aina *et al.* 2015; Fang *et al.* 2020). Likewise, seeds and leaves of cultivated peanut have been employed for hairy root induction (Guimarães *et al.* 2017; Raul and Sinharoy 2022).

2.2. Tissue culture regeneration

Wild peanuts typically yield few seeds, making them suboptimal as explants for *in vitro* regeneration. Leaves and leaflets are thus preferred for regenerating wild *Arachis* species. These tissues undergo dedifferentiation to

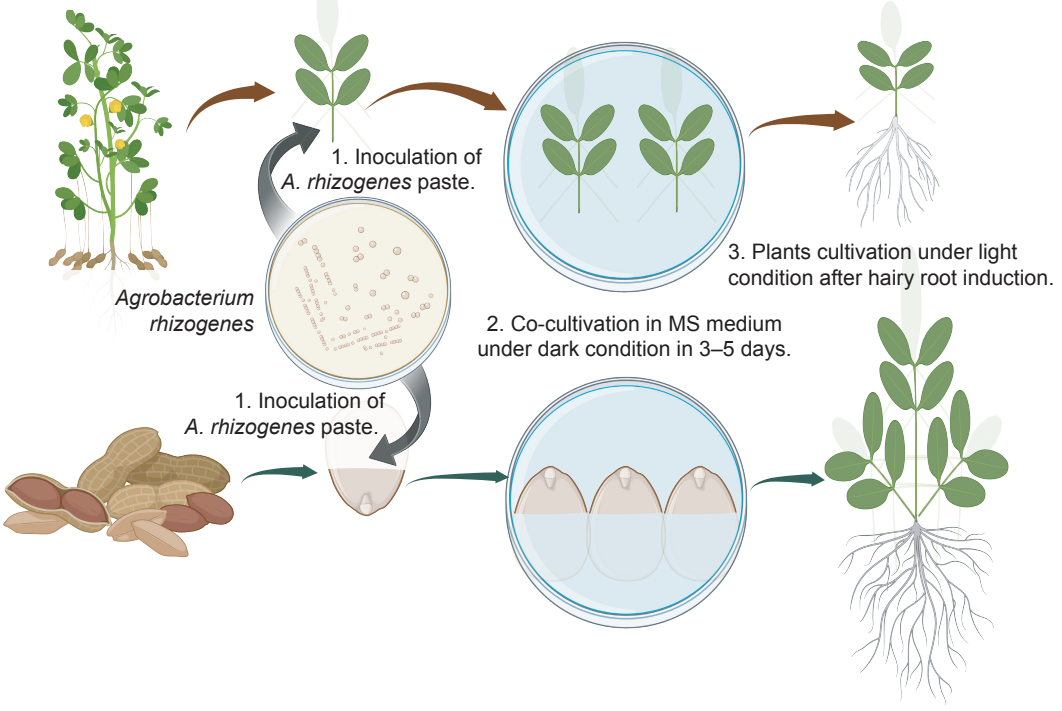


Fig. 1 Hairy root induction of *Arachis* species. Illustrations were obtained from BioRender. Protocol follows methods described in previous studies by Guimarães *et al*. (2017) and Raul and Sinharoy (2022). MS, Murashige and Skoog.

form callus, which subsequently redifferentiates into whole plants — a process known as tissue culture regeneration. Calluses from leaves and leaflets of *A. correntina*, *A. glabrata*, *A. paraguariensis*, *A. pintoi*, and *A. villosa* have been induced to develop into plantlets. Both mature and immature leaf tissues of *A. correntina* can initiate callus formation (Mroginski *et al.* 2004; Vidoz *et al.* 2006). The first expanded leaflet of *A. glabrata* has been used for callus induction (Vidoz *et al.* 2004; Dolce *et al.* 2018). Additionally, leaf-derived protoplasts of *A. paraguariensis* have formed calluses capable of regenerating plants (Li *et al.* 1993). Shoot apices and immature leaves of *A. pintoi* can also generate callus for regeneration, though diploid and triploid accessions require different hormone concentrations (Rey *et al.* 2000; Rey and Mroginski 2006; Carvalho *et al.* 2010). Immature leaves of *A. villosa* are similarly suitable for callus induction (Fontana *et al.* 2009). Although hormone concentrations vary across species, callus regeneration efficiency depends on leaf tissue age, with younger tissues yielding higher rates. Besides leaf tissues, embryos, cotyledonary nodes, and stem nodules can also serve as callus-inducing explants in wild peanuts (Gagliardi *et al.* 2002; de Sousa-Machado *et al.* 2018).

In cultivated peanut, callus induction employs diverse explants. The regeneration rate from mature zygotic embryo axes is lower in the variety *fastigiata* compared to *hypogaea* and *vulgaris* (McKently 1995). Embryos of cultivars Golden and BARI 2001 have been used for callus induction in MS medium containing BAP and NAA, respectively (Ahmad *et al.* 2020). Immature leaves and leaflets yield highly regenerative calluses in multiple peanut varieties (Baker and Wetzstein 1998; Chengalrayan *et al.* 1998; Pestana *et al.* 1999; Venkatachalam *et al.* 1999). Cotyledonary segments of cultivars VRI-2 and VRI-3 have successfully induced callus (Palanivel *et al.* 2002). Thidiazuron promotes callus formation from immature cotyledons and embryo axes (Gill and Ozias-Akins 1999). Hypocotyls of cultivars Co-5 and Co-7 have also been used for callus induction and subsequent plant regeneration (Muthusamy *et al.* 2007).

3. Genetic transformation of *Arachis* plants

Genetic engineering is essential for crop improvement through modulation of key gene expression. Peanut genetic transformation was first reported in the 1990s (Lacorte *et al.* 1991). To date, several methods, including biolistic delivery, virus-induced gene silencing (VIGS), hairy root transformation, pollen tube transformation, and tissue culture-based transformation, have been applied in peanut transgenic research (Table 1). Transgenic peanut lines exhibit enhanced abiotic and biotic stress tolerance and elevated oleic acid content relative to controls (Appendix A). These findings indicate that, except for *A. rhizogenes* in hairy root transformation, no specific vectors or *Agrobacterium* strains are consistently used across methods (Appendix A).

3.1. Non-tissue culture transformation

Biolistic DNA transfer The biolistic method was among the earliest techniques for introducing genes into peanuts to generate transgenic lines. Leaf tissues, leaflets, embryos, and cotyledons serve as explants (Clemente *et al.* 1992; Ozias-Akins *et al.* 1993; Deng *et al.* 2001; Chu *et al.* 2013). Target genes are delivered into plant cells *via* high-velocity microprojectiles coated with plasmid DNA (Klein *et al.* 1987). However, this method faces limitations such as high explant demand, low-copy integration, high cost, DNA mutations, and frequent generation of multicopy transgenic lines. Despite these drawbacks, numerous transgenic lines have been produced. Overexpression of *mercuric ion reductase*, *nonheme chloroperoxidase*, and *chitinase via* biolistics improved tolerance to $HgCl_2$, resistance to *Aspergillus flavus*, and resistance to leaf spot, respectively (Yang *et al.* 2003; Niu *et al.* 2009; Hassan *et al.* 2016).

Cotyledon/Cotyledonary node transformation Cotyledon or cotyledonary node is widely used for peanut genetic transformation due to its high efficiency and genotype independence (Hsieh *et al.* 2017; Huai *et al.* 2023). This method relies on the *in planta* regeneration system and co-cultivation of explants with *Agrobacterium* (Fig. 2). Numerous transgenic peanut lines with enhanced abiotic

Table 1 Genetic transformation of *Arachis* plants

Method	Explant	Agrobacterium	Peanut genotype	Gene editing	References
Biolistic DNA transfer	Callus, cotyledon, embryo, leaflet	No	Independent	Applicable	Clemente *et al.* (1992); Ozias-Akins *et al.* (1993); Deng *et al.* (2001); Chu *et al.* (2013)
Cotyledonary node transformation	Cotyledonary node	*Agrobacterium tumefaciens*	Independent	Inapplicable	Venkatachalam *et al.* (1998); Huai *et al.* (2023)
Hairy root transformation	Cotyledon, hypocotyl, leaf, leaflet, seed, seedling	*Agrobacterium rhizogenes*	Independent	Applicable	Guimarães *et al.* (2017); Raul and Sinharoy (2022)
Node injection	Node	*Agrobacterium tumefaciens*	Independent	Applicable	Han *et al.* (2023)
Pollen tube transformation	Flower	*Agrobacterium tumefaciens*	Independent	Applicable	Zhou *et al.* (2023)
Protoplast transformation	Leaflet, seedling	No	Independent	Applicable	Biswas *et al.* (2022)
Tissue culture transformation	Cotyledon, hypocotyl, leaf, leaflet	*Agrobacterium tumefaciens*	Dependnet	Inapplicable	Franklin *et al.* (1993); Cheng *et al.* (1996); Egnin *et al.* (1998)
Virus induced gene silence	Seedling	*Agrobacterium tumefaciens*	Dependnet	Inapplicable	Luo *et al.* (2021); Liao *et al.* (2023)

and biotic stress tolerance have been developed using this approach (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021).

Hairy root transformation Hairy root transformation is a popular, genotype-independent method that uses *A. rhizogenes* instead of *A. tumefaciens* as the medium. It enables only transient expression of the target gene. Seeds and leaves are commonly used as explants for *Arachis* species (Fig. 1). Previous studies show that hairy roots derived from leaf tissues retain leaf-like characteristics (Liu *et al.* 2023). Nevertheless, the method is valuable for studying root biology (Li L *et al.* 2023). Knockout of a nodule-specific gene *via* hairy root transformation resulted in abnormal nodule morphology in the cultivar Tifrunner (Raul and Sinharoy 2022). Overexpression of *AdEXLB8* gene from *A. duranensis* enhanced nematode resistance in cultivar Runner IAC-866 (Guimarães *et al.* 2017). *AhAREB1* positively regulates drought tolerance by reducing reactive oxygen species accumulation in hairy roots (Liu *et al.* 2016).

Pollen tube transformation Pollen tube transformation is a novel method for peanut genetic modification (Fig. 3). It resembles *Agrobacterium*-mediated pollen tube transformation in *A. thaliana*. In peanuts, *A. tumefaciens* is infiltrated into the carina (or keel) petals enclosing the ovary and stamens (Zhou *et al.* 2023). This method exhibits high transformation efficiency and is compatible with gene editing technologies. Transgenic lines overexpressing *AhBI-1* with improved root and shoot development have been obtained (Zhou *et al.* 2023). Similarly, pollen tube transformation has generated transgenic lines with enhanced salt tolerance through overexpression of *AhWRKY75* (Zhu *et al.* 2021).

Virus-induced gene silencing There is ongoing debate regarding the applicability of VIGS in peanut transformation. While Senthil-Kumar *et al.* (2007) reported failure of the VIGS system in peanut, recent studies demonstrate successful application in Chinese cultivars such as Fh and Zhonghua 2, suggesting genotype dependency. VIGS-mediated knockdown of *AhABI4* enhanced drought tolerance in cultivar Fh (Luo *et al.* 2021). Similarly, silencing *AhRUVBL2* in shoots of cultivar Fh reduced stem length, lateral root length, leaf size, and yield (Yang *et al.* 2023). The VIGS system has also produced *AhRPN1a*-like mutant lines in cultivar Zhonghua 2, which showed reduced *AhMC1* expression, increased root growth, and decreased aluminum-induced programmed cell death and 26S proteasomal activity (Liao *et al.* 2023).

3.2. Tissue culture transformation

Tissue culture transformation is a classical method but is highly genotype-dependent, limiting its utility. To date, only a few cultivars have been successfully transformed using this approach (Fig. 4). The hypocotyl of cultivar Okrun was

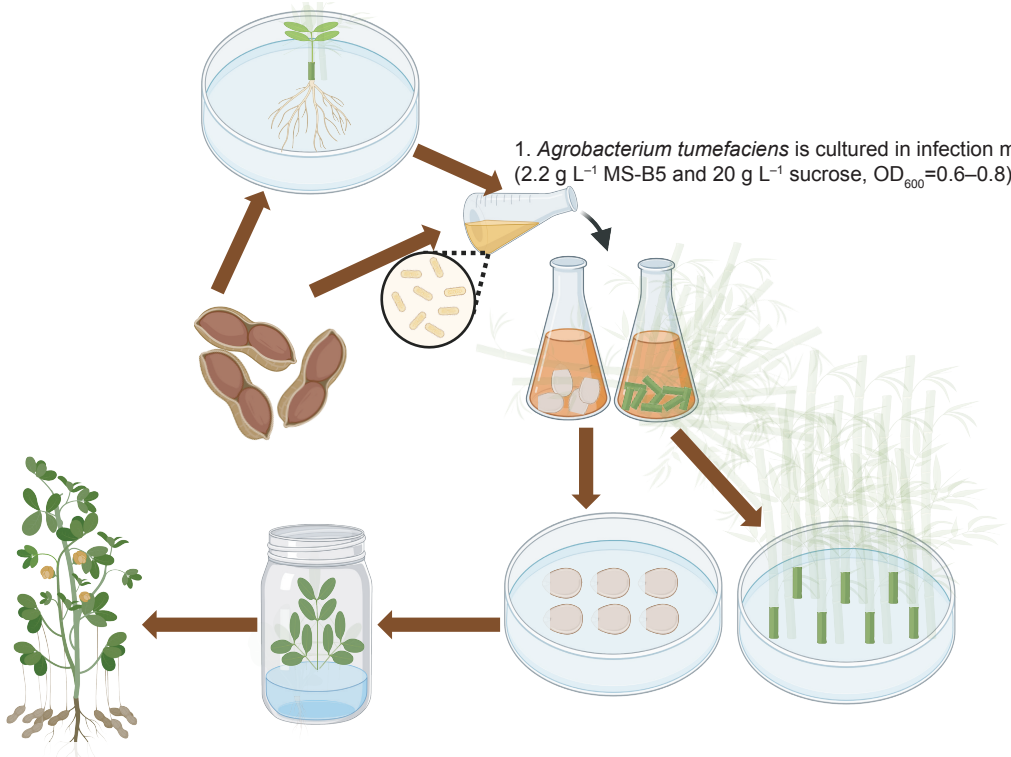


Fig. 2 Cotyledon and cotyledonary node transformation of *Arachis* species. Illustrations were obtained from BioRender. The protocol refers to previous studies by Huai *et al.* (2023) and Banavath *et al.* (2018).

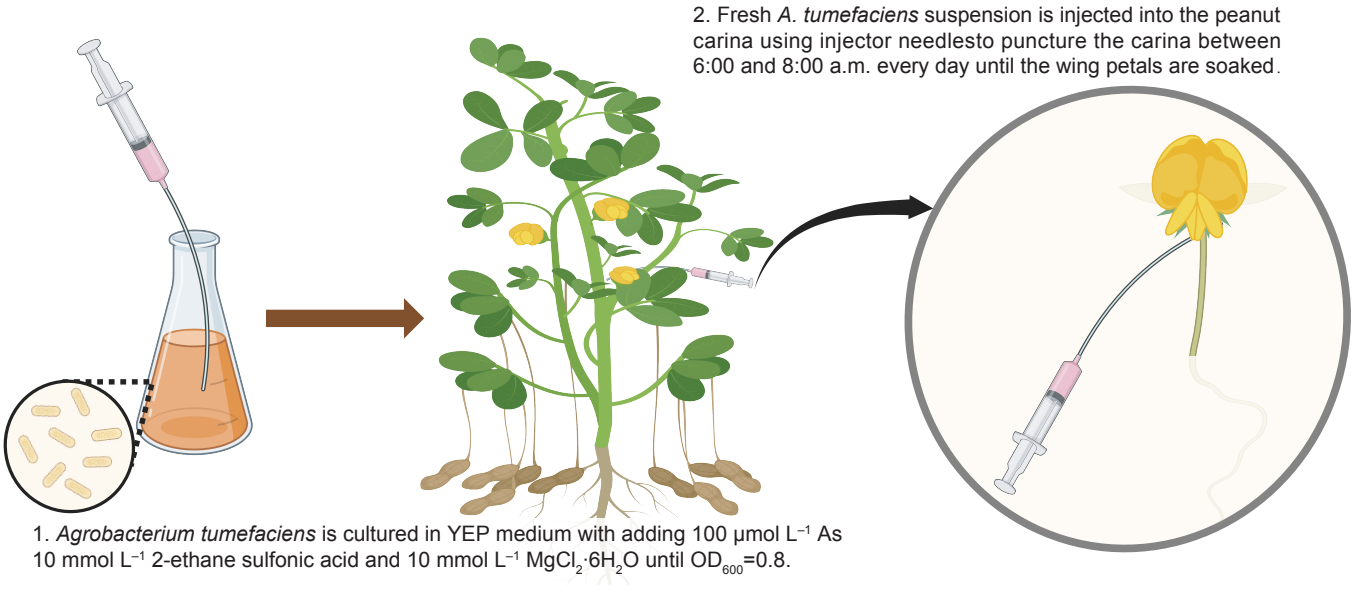


Fig. 3 Pollen tube transformation in *Arachis* species. Illustrations were obtained from BioRender. Protocol follows a previous study by Zhou *et al*. (2023). YEP, yeast extract peptone; As, acetosyringone.

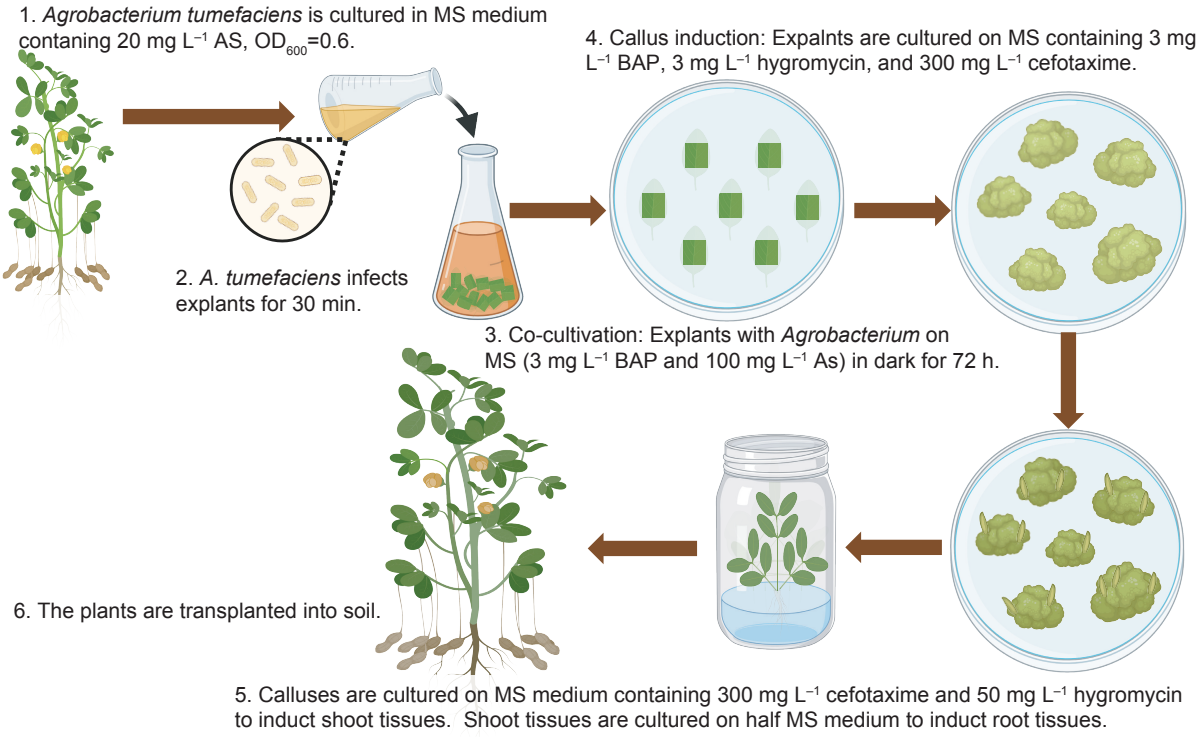


Fig. 4 Tissue culture transformation of *Arachis* species. Illustrations were obtained from BioRender. Protocol refers to a previous study by Chen *et al*. (2015). MS, Murashige and Skoog; As, acetosyringone; BAP, 6-benzylaminopurine.

transformed using *A. tumefaciens* strain EHA101, and the resulting calluses were regenerated into transgenic plants (Franklin *et al.* 1993). Leaf tissues of cultivar New Mexico Valencia A were co-cultured with *A. tumefaciens* strain EHA105 to introduce the pBI121 vector containing the *β*-glucuronidase (GUS) gene (Cheng *et al.* 1996). Different explants exhibit varying transformation efficiencies. Cotyledons generally show higher efficiency than leaf tissues (Egnin *et al.* 1998). However, the use of tissue culture transformation in peanut has declined significantly in recent years.

4. Application of CRISPR/Cas9 technology in peanut genetic transformation

Although peanut is considered recalcitrant to conventional genetic transformation due to low efficiency, gene editing technologies like CRISPR/Cas9 have been successfully applied. Recently, *AhALS2-A* and *AhALS2-B* genes were edited in cultivar Yuhua 9326 using the biolistic DNA delivery

method, resulting in transgenic lines with improved herbicide resistance (Shi *et al.* 2023). The CRISPR/Cas9 system preferentially represses the *FAD2* gene in subgenome A over subgenome B when delivered *via* hairy root transformation (Yuan *et al.* 2019). Transgenic lines with high oleic acid content have been generated by silencing *AhFAD2* using CRISPR/Cas9 (Neelakandan *et al.* 2022a, b, c). In cultivar Huayu 23, CRISPR/Cas9 was used to insert an adenine base at the 422nd site of *FAD2B via* node infiltration, producing high-oleic lines (Han *et al.* 2023). Pollen tube transformation has also enabled CRISPR/Cas9-mediated knockout of *AhFatB* in cultivar HY23, reducing palmitic acid and increasing oleic acid content (Tang *et al.* 2022). Similarly, *AhMULE9A* was knocked out in cultivar ZH-2 to enhance aluminum tolerance (Li A *et al.* 2023). Protoplast transformation has been used for gene editing. For example, *Ara h2* was silenced *via* CRISPR/Cas9 in protoplasts (Biswas *et al.* 2022).

5. Validation of transformed plants

Multiple methods are used to verify genetically transformed plants, including PCR, GUS staining, quantitative real-time PCR, Northern blot, Southern blot, and Western blot (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021). Phenotypic evaluation is also employed to assess transformation success. For instance, transgenic peanuts expressing genes for stress tolerance or high oleic acid content are evaluated for these traits (Krishna *et al.* 2015; Mallikarjuna *et al.* 2016; Lamboro *et al.* 2021). However, these markers and phenotypes represent indirect evidence. Recently, the *Tc1* (testa color 1) gene, controlling testa color, was identified in RIL populations using quantitative trait locus sequencing (QTL-seq) and bulked segregant analysis sequencing (BSA-seq) (Zhao *et al.* 2020). Overexpression of *Tc1* in tobacco produced purple plant in transgenic lines (Zhao *et al.* 2020), suggesting its potential as direct visual marker for transformation validation.

6. Conclusion

The lack of a robust genetic transformation protocol for *Arachis* species hinders gene function and molecular mechanism studies. Although several studies have reported successful gene transfer into *Arachis*, a significant contradiction persists between transformation capability and functional gene validation. This review summarizes *in vitro* regeneration and genetic transformation methods in *Arachis* to advance understanding of transgenic research. We find that *in vitro* regeneration methods can support *Agrobacterium*-mediated transformation. However, tissue culture transformation remains highly genotype-dependent, restricting broader application. Few recent reports describe *Agrobacterium*-mediated tissue culture transformation, indicating technical barriers. Biolistic and protoplast transformation have been used but face limitations that limit widespread adoption. *Agrobacterium*-mediated hairy root transformation has emerged as a major tool for root biology studies in *Arachis*.

Furthermore, *Agrobacterium*-mediated *in planta* transformation has notable limitations. It verifies functions of known genes but cannot identify novel genes from RIL populations. Breeders have developed many RILs to map genes for testa color and pod size, yet a reliable transformation system is needed to validate these candidates.

Standard detection methods like PCR and southern blot often yield false positives. Thus, visible phenotypic markers, such as pigmentation changes, are ideal for confirming transformation. For example, using *Tc1* as a marker gene could enhance the reliability of genetic transformation in *Arachis*.

In summary, the reliability of genetic transformation in *Arachis* species requires further validation, and a rapid, efficient transformation system must be established to support functional gene studies.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing processs

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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