A protocol for the generation of *Arachis hypogaea* composite plants: A valuable tool for the functional study of mycorrhizal symbiosis

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**Abstract**

**Premise:** *Agrobacterium rhizogenes*–induced hairy root systems are one of the most preferred and versatile systems for the functional characterization of genes. The use of hairy root systems is a rapid and convenient alternative for studying root biology, biotic and abiotic stresses, and root symbiosis in in vitro recalcitrant legume species such as *Arachis hypogaea*.

**Methods and Results:** We present a rapid, simplified method for the generation of composite *A. hypogaea* plants with transgenic hairy roots. We demonstrate a technique of hairy root induction mediated by *A. rhizogenes* from young *A. hypogaea* shoots. The efficacy of the system for producing transgenic roots is demonstrated using an enhanced green fluorescent protein (eGFP) expression vector. Furthermore, the application of the system for studying root branching is shown using the auxin-responsive marker *DR5* promoter fused to β-glucuronidase (GUS). Finally, the success of the hairy root system for root symbiotic studies is illustrated by inoculating hairy roots with arbuscular mycorrhizal fungi.

**Conclusions:** In this study, we have developed a rapid, efficient, and cost-effective composite plant protocol for *A. hypogaea* that is particularly effective for root-related studies and for the validation of candidate genes in *A. hypogaea* during mycorrhizal symbiosis.

**KEYWORDS**

*Agrobacterium rhizogenes*, *Arachis hypogaea*, arbuscular mycorrhizal fungal symbiosis, groundnut, hairy roots, K599, peanut, promoter analysis

Peanut (also called groundnut; *Arachis hypogaea* L.), a member of the Leguminosae family, is native to South America and is an important oil crop, as well as a source of proteins, calcium, iron, vitamin B complex compounds (e.g., thiamine, riboflavin, niacin), and vitamin A. *Arachis hypogaea* is distributed across tropical and temperate regions worldwide and is cultivated in more than 100 countries, with a total worldwide production of 48.8 million metric tons in 2019 (Food and Agriculture Organization of the United Nations, 2019). However, peanut production suffers significant losses, predominantly from insects, nematodes, and diseases. Among the alternative approaches to control these problems, an association with arbuscular mycorrhizal (AM) fungi can provide protection against nematodes (Campos, 2020) and reduce pathogen severity (Sikes, 2010).

In recent years, developments including the sequencing of the peanut genome (Bertioli et al., 2019; Zhuang et al., 2019) and the creation of the genetics and genomics database PeanutBase (Dash et al., 2016) have accelerated breeding advancements in this crop. At the same time, RNA-Seq experiments have produced specific transcriptomic profiles of mycorrhized peanut roots (Cui et al., 2019). However, in vitro recalcitrance (i.e., the inability of plant cells, tissues,
and organs to respond to tissue culture manipulations) has impeded research related to functional characterization of candidate genes in this legume. The soil bacterium *Agrobacterium rhizogenes* offers a fast and reliable alternative to tissue culture in such recalcitrant legumes, producing composite plants with transgenic roots and nontransgenic shoots. This technique has already been adopted for more than 100 plant species (Veena and Taylor, 2007) and provides a time-saving alternative to the often laborious and inefficient process of stable genetic transformations produced via tissue culture (Taylor et al., 2006). By eliminating the tissue culture step, the ex vitro composite plant method provides a cost-effective and highly successful approach that can be used in studies related to root biology, abiotic stress, biotic stress, and root symbiosis in *A. hypogaea*.

Previous molecular research has established peanut hairy root cultures as an alternative approach for producing commercially important secondary metabolites (e.g., the use of hairy roots induced from peanut cotyledonary node tissue [Medina-Bolivar et al., 2007] and leaf explants [Kim et al., 2009]). Studies using transformed peanut hairy roots have also been used to examine the genes involved in root nodule symbiosis interactions (Akasaka et al., 1998; Sinharoy et al., 2009), plant–nematode interactions in detached peanut leaves (Chu et al., 2014; Guimaraes et al., 2017), and drought tolerance (Liu et al., 2016). To date, no reports exist regarding the use of this system to study AM symbiosis in peanut.

Here, we present a simple, rapid, and efficient method that does not involve subculturing after the explants of *A. hypogaea* are inoculated with *A. rhizogenes*. The efficiency of the transformation is demonstrated by expressing an enhanced green fluorescent protein (eGFP), a visual marker protein that delivers green fluorescence in hairy roots. Furthermore, the efficacy of *A. rhizogenes*–induced hairy roots for analyzing root biology, root biochemistry, and root symbiosis mediated by AM fungi is demonstrated by comparison with the respective controls. Overall, the protocol described here could be adopted for a wide variety of root-related analyses and for the functional characterization of genes in *A. hypogaea* during mycorrhization.

**METHODS AND RESULTS**

**Sterilization and germination of seeds**

*Arachis hypogaea* cv. Tlaxmalac seeds were used in this study. The seeds were surface sterilized by immersion in absolute ethanol for 1 min and in 10% (v/v) sodium hypochlorite for 5 min, followed by five washes with sterile distilled water. Next, the seeds were imbibed in ~40 mL of sterile distilled water for 8 h under low-speed (25–30 rpm) swirling at 25°C (for high water uptake and uniform seed imbibition). The seeds were then germinated on sterile filter paper moistened with B&D media (Broughton and Dilworth, 1971; Appendix S1) in darkness for two days at 28°C. Two-day-old germinated seeds were planted in pots containing sterile vermiculite and maintained in a growth chamber under a 16 h light : 8 h dark photoperiod and 65% relative humidity at 28 ± 1°C. The plants were checked daily and irrigated with B&D media as needed (Figure 1A).

**Protocol for the generation of composite *A. hypogaea* plants**

To obtain *A. hypogaea* composite plants by *A. rhizogenes*–mediated root transformation, we used the *A. rhizogenes* K599 strain to infect *A. hypogaea*. A pH7WG2D.1 vector (Karimi et al., 2002) containing an eGFP reporter gene driven by the constitutively active CaMV 35S promoter was transformed into the K599 strain by the electroporation method with the following settings: 1.8 kV, 25 μF, and 200 Ω. Positive colonies (pH7WG2D.1-35-eGFP) were selected on Luria–Bertani agar plates consisting of 100 mg/L spectinomycin, and glycerol stocks were prepared and stored at −80°C. The day before the transformation, 150 μL of K599/pH7WG2D.1-35-eGFP culture harboring the binary vector was streaked onto solid Luria–Bertani media containing 100 mg/L spectinomycin and grown at 28°C overnight.

The hairy root transformation protocol was tested initially to check the effects of the explant stage, i.e., shoot tip with *n* (2, 4, or 6) fully opened trifoliate leaves, and plant growth media on the transformation efficiency. We observed significantly high cotransformation efficiency for explants with two fully opened trifoliate leaves compared to four and six leaves (Table 1). Similarly, we observed that, compared with perlite, sterile vermiculite moistened with B&D nutrient media resulted in high cotransformation efficiency (Table 1). These preliminary experiments indicated that the explants with two fully opened trifoliate leaves maintained on sterile vermiculite as plant growth media yielded high transformation efficiency for *A. hypogaea*.

Young and fully open trifoliate leaves (*n*2) along the main stem (explants; Figure 1B) were cut at a 45° angle using a scalpel (Figure 1C). The cut end of the explant was scraped onto a freshly prepared K599/pH7WG2D.1-35-eGFP cell mat (Figure 1D), and the inoculated cut end was kept immersed at a depth of 3 cm in sterile vermiculite moistened with B&D media in a tightly capped tissue culture jar. The jars were incubated in a 16 h light : 8 h dark photoperiod in a growth chamber at 28 ± 1°C for 10–12 d. No separate coculture (for *virulence* gene activation and attachment of *A. rhizogenes* to plant cells) was required for this method. Our results showed that, within 6–7 d post-inoculation, a mass of callus started to form at the wounding site (Figure 1E), and by 10–12 d post-inoculation, hairy roots 2–3 cm in

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length had emerged (Figure 1F). When visualized under a stereofluorescence microscope, the transgenic hairy roots showed strong GFP expression, whereas no such GFP expression was found in the nontransgenic roots (Figure 1G, H). At this stage, the nontransgenic roots were removed, and the composite *Arachis hypogaea* plants were transplanted into pots containing sterile vermiculite (Figure 1I).

## Gene functional studies using peanut transgenic hairy roots

### Plant promoter analysis

To evaluate the suitability of the *A. rhizogenes*–mediated hairy root transformation technique for promoter activity studies of genes involved in root development, we utilized the

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**TABLE 1** Transformation efficiency of *Arachis hypogaea* explants with *Agrobacterium rhizogenes* K599 strain

| *A. hypogaea* cultivar | *A. rhizogenes* strain | Explant stage\(^a\) (n) | Plant growth medium | No. of plants (transgenic roots/plant)\(^b\) | Co-transformation efficiency (%)\(^c,d\) |
|------------------------|------------------------|------------------------|---------------------|---------------------------------|----------------------------------|
| Tlaxmalac              | K599                   | 2                      | Vermiculite         | 45 (4.20)                       | 93.3\(^A\)                      |
|                        |                        | 2                      | Perlite             | 40 (3.48)                       | 82.5\(^B\)                      |
|                        |                        | 4                      | Vermiculite         | 41 (3.63)                       | 80.5\(^B\)                      |
|                        |                        | 4                      | Perlite             | 41 (3.50)                       | 73.2\(^C\)                      |
|                        |                        | 6                      | Vermiculite         | 25 (4.32)                       | 60.0\(^D\)                      |
|                        |                        | 6                      | Perlite             | 29 (3.90)                       | 41.4\(^E\)                      |

\(^a\) Shoot tip with \(n\) number of fully opened trifoliate leaves.

\(^b\) Average number of transgenic roots/composite plant.

\(^c\) Transformation efficiency calculated at 12 days post-infection (incubated in 16 h light : 8 h dark photoperiod at 28 ± 1°C) as the percentage of plants presenting at least one fluorescent root over the total number of infected plants.

\(^d\) Uppercase letters denote significant differences at the level of \(P = 0.05\).
above-demonstrated approach to study the promoter activities of DR5 in A. hypogaea hairy roots. DR5 is an established marker of the auxin response and an indirect marker of auxin accumulation (Sabatini et al., 1999). pCAMBIA1300 harboring the synthetic promoter DR5::GUS (proDR5::GUS) in A. rhizogenes K599 was used to induce transgenic hairy roots in A. hypogaea; the same strain was previously used by Liu et al. (2016) and Guimaraes et al. (2017). An empty vector without the DR5 promoter served as the control. At two weeks post-infection, the A. hypogaea transgenic roots (those of both proDR5::GUS and the control plants) were assessed to determine the spatiotemporal expression pattern of the DR5 promoter through histochemical β-glucuronidase (GUS) assays. The transgenic roots were incubated in freshly prepared GUS buffer consisting of 1 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl beta-D-glucuronide cyclohexamine salt) at 37°C for 12 h in the dark (Jefferson et al., 1987). We observed transgenic roots of proDR5::GUS showing strong GUS expression in the primary root tips and lateral root tips (Figure 2C, D). In the mature root zones, DR5 promoter activity was also observed in the vascular tissues during lateral root development. However, no such GUS expression was observed in the hairy roots expressing the empty vector (Figure 2A, B). In other crops, such as cucumber (Fan et al., 2020a) and soybean (Fan et al., 2020b), a similar approach was adopted to express a nucleolar WD40-repeat promoter YAO::GUS using hairy roots. Therefore, this method provides a rapid means to analyze promoter function.

Effects of arbuscular mycorrhizal fungal colonization on root growth of A. hypogaea

Root systems in different plant species respond variably to AM colonization (reviewed in Fusconi, 2014). Herein,
FIGURE 3  Effects of AM fungal colonization on the growth of hairy roots of *Arachis hypogaea*. (A) Uninoculated hairy roots free of AM fungi. (B, C) AM fungus–inoculated hairy roots showing fungal structures such as (B) arbuscules (ar) and intraradical hyphae (irh), and (C) a vesicle (marked with an arrow). Scale bars = A: 50 µm; B, C: 20 µm

FIGURE 4  Quantification of AM fungal colonization and root growth of *Arachis hypogaea* hairy roots. (A) Percent mycorrhizal root length colonization of wild-type non-transgenic roots and transgenic roots expressing pH7WG2D.1-35-eGFP at two weeks post-inoculation. (B, C) Root quantitative data of growth parameters including (B) primary root length and (C) lateral root density at two weeks post-inoculation. The data are the means of three biological replicates (*n* > 18). The statistical significance of differences between uninoculated and AM fungus–inoculated hairy roots was determined using an unpaired two-tailed Student’s *t*-test (*P* < 0.05; **P** < 0.01), and the error bars represent the means plus SEMs. AU, arbitrary unit
we analyzed the effects of AM fungal colonization on the root growth of *A. hypogaea* composite plants. First, we generated hairy roots expressing the pH7WG2D.1-35cGFP construct; these hairy roots were then inoculated with the AM fungus *Rhizophagus irregularis* (500 spores/plant) (Symplanta, Darmstadt, Germany) and irrigated twice weekly with half-strength B&D solution consisting of a low concentration of potassium phosphate (10 µM, K2HPO4) to promote AM colonization (Arthikala et al., 2013). Both uninoculated and AM fungus–inoculated roots were harvested at two weeks
| Parameter                               | Present work                          | Akasaka et al. (1998) | Kim et al. (2009) | Sinharoy et al. (2009) | Liu et al. (2016) | Guimaraes et al. (2017) |
|----------------------------------------|---------------------------------------|-----------------------|-------------------|------------------------|-------------------|-------------------------|
| Source of explant                      | Greenhouse-grown plants, surface sterilized | Surface-sterilized embryonic axis | In vitro-grown plants | In vitro-grown plants | Surface-sterilized embryonic axis | Greenhouse-grown plants |
| Explant                                | Shoot tip                             | Embryonic axis        | Detached leaf      | Embryonic axis         | Petiole           | Detached leaf           |
| Co-cultivation                         | Scraping *A. rhizogenes* (K599) culture on the cut end of the shoot tip | Co-cultivation with *A. rhizogenes* (MAFF-02-10266) with acetosyringone at 10 mg/L | Co-cultivation with *A. rhizogenes* cultures (15834, A4, R1000, R1200, and R1601) | Co-cultivation with *A. rhizogenes* (R1000) | Co-cultivation with *A. rhizogenes* (K599) with acetosyringone at 10 mg/L | Injecting with *A. rhizogenes* (K599) on the petiole |
| Post-cultivation sterilization         | Not required                          | Post-co-cultivation sterilization of the explants and treatment with cefotaxime at 200 mg/L | Not required | Post-co-cultivation sterilization of the explants | Post-co-cultivation sterilization of the explants and treatment with acetosyringone at 10 mg/L | Not required |
| Growth media                           | Sterile vermiculite                   | 4 g/L gellan gum-solidified 1/2 MS media | MS media | MS media | MS media | Wet filter paper until the hairy roots emerge |
| Efficiency                             | 93.3%                                 | 62%                   | Varied across the bacterial strain | 95% | 91% | 90% |
| Time taken for hairy root emergence    | 10–12 days                            | 20 days               | 30 days           | 17–20 days            | Two weeks        | 20 days                 |

Note: MS, Murashige and Skoog
post-inoculation, and a small portion of the roots were stained with trypan blue according to the methods of McGonigle et al. (1990). Root fragments (1 cm in length) were subsequently fixed in FAA solution (formaldehyde, ethanol, and acetic acid, 10% : 50% : 5% + 35% water) overnight. After several washes, the roots were treated with 5% KOH, heated at 90°C for 1 h, washed three times in distilled water, stained in trypan blue dye (composed of 0.05% trypan blue in acetic glycerol), and boiled at 90°C for 1 h. The stained roots were then examined to visualize AM fungal structures, such as intraradical hyphae, arbuscules, and vesicles, using a light microscope (DMLB bright-field microscope; Leica, Wetzlar, Germany). The remaining portion of each root sample was used to determine the root growth parameters. Our results showed that the AM fungus–inoculated hairy roots had intraradical hyphae, several mature arbuscules, and vesicles (Figure 3B, C), indicating successful mycorrhizal colonization. The quantification of root length colonization of AM fungi showed 45% and 46.9% in wild-type and transgenic hairy roots (pH7WG2D.1-35-eGFP) of peanut, respectively (Figure 4A), indicating no significant difference in fungal colonization between transgenic and non-transgenic roots. In contrast, neither fungal structures nor colonization were observed in the uninoculated hairy roots (Figure 3A). Previously, Ho-Plágaro et al. (2018) used a similar technique in tomato and demonstrated that it is an easy and fast protocol for mycorrhizal studies and screening of candidate genes for involvement in AM fungal symbiosis. The quantitative data show that root growth parameters, such as the length of the primary root, were increased slightly but significantly in the AM fungus–inoculated hairy roots compared to the uninoculated roots (Figure 4B). However, lateral root density was significantly higher for the AM fungus–inoculated roots than the uninoculated hairy roots (Figure 4C). The lateral root density was calculated using the formula $D = LR/L$, where D is the density of lateral roots, LR is the number of lateral roots, and $L$’ is the length of the main root between the first and last lateral root (Dubrovsky et al., 2006).

Previous work has revealed that the accumulation of reactive oxygen species (ROS), namely, superoxides and hydrogen peroxide, is crucial for lateral root primordium formation in Arabidopsis Heynh. (Tyburski et al., 2009) and Phaseolus L. (Montiel et al., 2013; Arthikala et al., 2017). Because AM fungal colonization significantly enhanced lateral root induction in A. hypogaea, we assessed superoxide and hydrogen peroxide accumulation in AM fungus–inoculated hairy roots and compared it with that in uninoculated hairy roots. The production of superoxide was determined by carefully placing the roots in 10 mM sodium phosphate buffer (pH 7.8) followed by vacuum infiltration for 1 h. The roots were then incubated in sodium phosphate buffer (10 mM) consisting of 0.1% nitro blue tetrazolium (NBT) until formazan precipitates were visible to the naked eye (Montiel et al., 2012). Accumulation of hydrogen peroxide was observed by placing the hairy roots in freshly prepared 1.25 mg/mL DAB (3,3'-diaminobenzidine)-HCl solution (pH 3.8) until brown DAB polymerization was observed (Ramsey et al., 2009). Our results showed high levels of superoxide accumulation in the lateral roots of both uninoculated and AM fungus–inoculated hairy roots (Figure 5A, C). Moreover, high hydrogen peroxide accumulation in the elongation zone of the hairy roots was observed (Figure 5B, D). These observations were further verified by quantifying the NBT and DAB staining intensity of hairy roots using ImageJ software (Schneider et al., 2012; Figure 6A, B).

Overall, we conclude that the proposed hairy root protocol can be adopted for symbiosis and ROS studies in A. hypogaea.

**CONCLUSIONS**

Genetic transformation techniques that are fast, efficient, and reproducible are essential for crop improvement. Limitations in transformation tools for large-seeded legumes, such as A. hypogaea, can be a significant barrier to advancing the understanding of gene function in this crop species. However, transient transformation techniques serve as an alternative for rapid gene functional characterization studies. In the present study, we established an efficient hairy root transformation system for A. hypogaea using A. rhizogenes by optimizing parameters, and further demonstrated the versatility of this technique for studies of gene function. Compared to the previously published methods (Table 2), this protocol has the following advantages: (1) it requires no additional coculture time; (2) it successfully produces hairy roots within 10–12 days post-inoculation; (3) it demonstrates transformation efficiency of 93.3%; (4) it uses inexpensive plant growth medium components such as vermiculite; and (5) it is suitable for a variety of rapid gene functional studies, such as spatiotemporal expression patterns of root-specific promoter analysis, plant–symbiont interaction analysis, root growth and development analysis, and biochemical assays related to ROS (i.e., superoxide and hydrogen peroxide) production. We hope this protocol can be used as a powerful and versatile tool for multiple gene functional studies in A. hypogaea and as a useful means for many other avenues of research, such as root biology/physiology studies, biosynthesis of plant-derived secondary metabolites, characterization of abiotic/biotic stress factors, and validation of candidate genes through forward or reverse genetic approaches.

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AUTHOR CONTRIBUTIONS
M.-K.A. conceived the research and designed the experiments; K.N. performed all the experiments. I.Z.-J. assisted in methodology and mycorrhizal cultures. M.-K.A. and K.N. acquired the funding, analyzed the data, and wrote the manuscript. All authors approved the final version of the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**Appendix S1.** Recipe for B&D nutrient solution.