Video Article

Generation of Composite Plants in *Medicago truncatula* used for Nodulation Assays

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Abstract

Similar to *Agrobacterium tumerfaciens, Agrobacterium rhizogenes* can transfer foreign DNAs into plant cells based on the autonomous root-inducing (Ri) plasmid. *A. rhizogenes* can cause hairy root formation on plant tissues and form composite plants after transformation. On these composite plants, some of the regenerated roots are transgenic, carrying the wild type T-DNA and the engineered binary vector; while the shoots are still non-transgenic, serving to provide energy and growth support. These hairy root composite plants will not produce transgenic seeds, but there are a number of important features that make these composite plants very useful in plant research. First, with a broad host range,*A. rhizogenes* can transform many plant species, especially dicots, allowing genetic engineering in a variety of species. Second, *A. rhizogenes* infect tissues and explants directly; no tissue cultures prior to transformation is necessary to obtain composite plants, making them ideal for transforming recalcitrant plant species. Moreover, transgenic root tissues can be generated in a matter of weeks. For *Medicago truncatula*, we can obtain transgenic roots in as short as three weeks, faster than normal floral dip Arabidopsis transformation. Overall, the hairy root composite plant technology is a versatile and useful tool to study gene functions and root related-phenotypes. Here we demonstrate how hairy root composite plants can be used to study plant-rhizobium interactions and nodulation in the difficult-to-transform species *M. truncatula*.

Video Link

The video component of this article can be found at http://www.jove.com/video/2633/

Protocol

The following protocol has been used to generate hairy root composite plants in a model legume species *M. truncatula*. Similar protocols have been adapted for at least eight plant species [1-4]. We used *M. truncatula* hairy root composite plants to study gene functions in root and nodule development. The protocol was separated into four sections: 1) preparing plant materials; 2) generating hairy root composite plants; 3) symbiotic *Rhizobia* infection; and 4) transgenic root identification. We used the binary vector containing the *green fluorescent protein* (*gfp*) gene as a reporter for screening transgenic root in composite plants [3]. Compared to antibiotics-based selection, GFP-based screening is fast, easy, and inexpensive. In our construct, the ER-expression-optimized *gfp* gene is driven by the super ubiquitin promoter, which has strong constitutive GFP signals in transgenic roots, allowing easy distinction between transgenic and non-transgenic roots.

1. Preparing Plant Materials

1. *M. truncautla*seeds are harvested from plants grown in a greenhouse (50% relative humidity, 16/8 h light/dark, 22/18°C day/night temperature). Mature seeds can be stored at 4°C.
2. Approximately 100 seeds are scarified in 10 ml concentrated sulfuric acid (95-98%, Sigma-Aldrich, MO) for 10 min with periodic shaking.
3. The seeds are rinsed 5-7 times with sterile water with gentle agitation.
4. The seeds are then soaked in water at room temperature for 6 hours and kept at 4°C for 36-48 hours to synchronize germination.
5. Approximately 20-30 seeds are spread on moist filter papers placed in a Petri dish. They are maintained at 22°C, with 16/8 h light/dark cycle, and 40 $\mu mol.m^{-2}.s^{-1}$ light intensity, for 5-6 days.
6. Germinated seedlings are transferred into soil and placed in greenhouse for a month.

2. Generating Hairy Root Composite Plants

1. Binary constructs carrying genes of interest were transformed into *A. rhizogenes* using standard protocols. We have engineered a set of binary vectors containing the *gfp* gene as a marker that facilitate screening of transgenic tissues. These vectors contain various promoters and all have Gateway cloning sites (Invitrogen, Carlsbad, CA) for over-expression or silencing of targeted genes [3,5].
2. *A. rhizogenes*is cultured in 50ml LB medium with appropriate antibiotic selection at 28°C for 16-24 hours.
3. The bacteria are collected and re-suspended to a final concentration of OD_{600}=0.3 in a nitrogen-free plant nutrient solution (Table 1 and [6]).

4. To support hairy root regeneration, a sterilized supporting matrix ("Rock Wool", Hummert International, Earth City, MO) is cut into plugs of approximately 3 cm^3. We place 4 plugs into one Petri dish.
5. A hole is poked on top of each plug using a pipette tip to facilitate the insertion of explants. *A. rhizogenes* (5 mL) is added to each plug.
6. A shoot section with 2-3 axillary buds is excised by a slanting cut. The explant is then inserted into the plug, and grown at 22°C for about three weeks. We keep the *Medicago* explants without watering for the first 10 days, then, water them with 5 ml nitrogen-free solution when necessary. We found that removing the apical meristem might decrease the formation of adventitious roots.

3. Symbiotic Rhizobia (*Sinorhizobium Meliloti*) Infection

1. After three weeks, some adventitious roots will emerge from the Rock Wool. The hairy root composite plants are transferred to sand or vermiculite (sterilized), with or without the Rock Wool. They are grown in greenhouse at 22°C, 16/8 h light/dark cycle, and 300 $\mu mol.m^{-2}.s^{-1}$ light intensity for one more week.
2. Prior to rhizobia infection, *S. meliloti* 1201 is cultured in 50 ml yeast extract-mannitol medium for 7 days at 30 °C (Table 2, and [6,7]).
3. Re-suspended rhizobia cells are diluted to a final concentration of OD_{600}=0.08 using nitrogen free nutrition solution. A 10-ml fresh suspension of*S. meliloti* was applied to each composite plant to induce nodule formation.

4. Transgenic Root Identification

1. Two weeks after rhizobia inoculation, the composite plants are up-rooted by washing in water. The roots can be easily separated from sands or perlite in water.
2. We place the hairy roots under a UV-microscope (Nikon SMZ1500, Excitation 460-500nm, Dichroic 505nm, Barrier more than 510nm). The transgenic roots are GFP positive and the adventitious roots are GFP negative. We then collect the roots according the GFP signal, and count the nodule numbers, measure the root length, and calculate lateral root density. The GFP negative roots are used as control.

5. Representative Result

In our experiment, the new hair roots regenerated from the explants in 2-3 weeks after *A. rhizogenes* innoculation. Under the UV-microscope, transgenic roots carrying the *gfp* gene show strong green fluorescence (Fig 1). The amount of regenerated roots and the portion of the GFP positive roots depend on the conditions of explants and the growth environment of composite plants.On average, 25% of the roots produced were transgenic hairy roots [3].To increase the transformation efficiency, 1) the plastic cover doom, as shown on the video, is enough to maintain the humidity in the growth tray for the first few days, and plants only require little water in the first few days. Excessive watering is detrimental to hairy root formation; 2) the concentration of *Agrobacterium* innoculant is important for transformation. Excessive amount of cells are not helpful to hairy root formation or nodule formation. For nodule formation, the watering solution needs to be nitrogen-free, otherwise, few nodules will form.

The hairy root composite plants can be generated using cotyledons or entact seedlings as the starting material. Only minor modifications of the above protocol are ncessary to generate hairy roots from other tissues [8]. Importantly, each hairy root is an independent transformation event. Therefore, the phenotypes observed in one composite plant are the sum of several transforamtion events that needed to be confirmed by repetitions in multiple composite plants

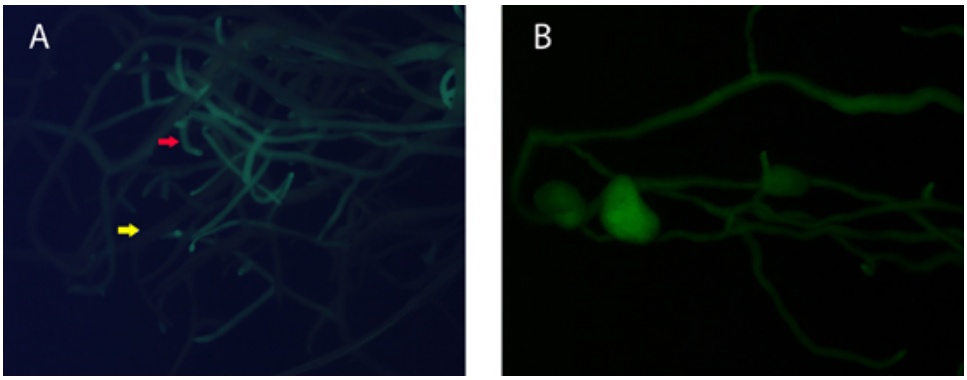

Figure 1. A. Transgenic roots can be sorted out from hairy roots. B. Nodules were formed in the hairy root composite plants. We placed 4-week-old *M. truncatula* hairy root plants under the UV-microscope and the GFP positive roots can be easily identified. (Red arrow: GFP root; Yellow arrow: non-GFP root)

Stock	g/200ml	ml stock/ liter
$MgSO_4.7H_2O$	12.3	2
$CaCl_2.2H_2O$	14.7	4
$K_2HPO_4.3H_2O$	6.8	1
K_2SO_4	11	4
Fe $Cl_3.6H_2O$	0.49	2.5
Micronutrients	See below	1

Micronutrients	g per liter
H_3BO_3	0.142
$MnSO_4.H_2O$	0.077

$ZnSO_4.7H_2O$	0.1725
$CuSO_4.5H_2O$	0.037
$NaMoO_4.H_2O$	0.024
$CoCl_2.H_2O$	0.0025
$NiSO_4$	0.001

Table 1. Nitogen-free nutrition solution

K_2HPO4	0.5g
NaCl	0.1g
$MgSO_4 \cdot 7H_2O$	0.2g
Yeast Extract	0.4g
Mannitol	10g
pH=6.8	

Table 2. yeast extract mannitol medium (per liter)

Discussion

Generating hairy root composite plant is a quick and easy method to obtain large quantities of transgenic material for many dicot species. Although this method cannot produce transgenic seeds, it can produce transgenic materials in a few weeks. The method is especially suitable for plants that have difficulty establishing tissue cultures or generating stable transformants. Over the years, we have used this technology to study gene functions, promoter functions, microRNAs, root and lateral root development, defense and abiotic stress responses, nodulation and other symbiotic processes, hormone responses, metabolic profiling, gene profiling, proteomic analysis, and other biological processes. The protocol is robust and replicable.

Disclosures

No conflicts of interest declared.

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