Agrobacterium rhizogenes-mediated root transformation

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Table of contents:

1. Introduction

In recent years rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious, genetically (Ri T-DNA) transformed roots. This leads to the production of so-called "composite plants" comprising a transgenic hairy root system attached to non-transformed shoots and leaves. *A. rhizogenes*-mediated transformation makes it possible to co-transform plant cells with more than one T-DNA at the same time. The T-DNA containing the transgene of interest in a disarmed binary vector is generally co-transformed with the resident *A. rhizogenes* Ri T-DNA containing the *root locus (rol)* genes (responsible for root proliferation). A key parameter in the exploitation of this system is the use of relatively low virulence *A. rhizogenes* strains such as Arqua-1 and K599, which elicit a limited number of Ri T-DNA-transformed roots with growth and morphology comparable to normal roots (Quandt *et al*., 1993; Collier *et al*., 2005). By comparison, strains such as MSU440 and R1000 are generally considered of higher virulence, and hence there is a risk that transformed roots will exhibit abnormal root structure and growth. Transformation of *M. truncatula* with *A. rhizogenes* Arqua-1 results in the production of composite plants with transgenic roots that are well-adapted for studies of root-specific interactions since they can be nodulated by *Sinorhizobium meliloti*, efficiently colonised by endomycorrhizal fungi and infected by pathogenic/parasitic organisms such as root nematodes (Boisson-Dernier *et al.*, 2001; Harrison *et al.*, 2002; Boisson-Dernier *et al.* 2005; see also Chapters "Rhizobial inoculation and nodulation of medicago truncatula" and "Inoculation and growth with mycorrhizal fungi"). In addition, transgenic root explants can be clonally propagated for studies of endomycorrhizal colonisation (e.g. Chabaud *et al.* 2002). Unfortunately such clonal explants cannot be used for nodulation studies since the *Rhizobium*/legume interaction requires aerial organs.

The major **advantage** of this approach is the rapidity and technical simplicity of *A. rhizogenes* transformation. Instead of the 4-6 months-time required to regenerate transgenic plants following *A. tumefaciens*-based transformation (see chapter "*Agrobacterium tumefaciens*-mediated transformation"), production of Ri T-DNA transgenic roots takes only a few weeks. Thus, this technique is particularly well adapted to RNAi-based approaches to study gene function (Limpens *et al*. 2004), performing functional promoter analyses (Boisson-Dernier *et al.* 2005) and as a means for rapidly evaluating the complementation of plant mutants with

candidate genes (e.g. Endre *et al.* 2002; Levy *et al*. 2004), when the genetic determinant of the corresponding mutation is root-determined.

The major **limitation** of this approach is that, since shoots are not transformed, sustained maintenance of composite plants is not possible either by vegetative propagation or self-fertilisation. Nevertheless, this limitation can now be circumvented since it has recently been shown that Ri T-DNA transformed *M. truncatula* roots can be successfully regenerated to plantlets (Crane *et al.*, 2006).

The following **two protocols** are presented in this chapter:

A – An *A. rhizogenes* transformation technique which leads to axenic composite plants. Excised transgenic roots can then be decontaminated (to remove *Agrobacterium*) and then propagated *in vitro*. The possibility of direct antibiotic (kanamycin) selection for roots expressing the co-transferred transgene of interest improves the overall efficiency of transformation.

B - An alternative *A. rhizogenes* transformation technique which leads to vigorous composite plant culture without employing axenic tissue culture facilities.

A - Protocol for axenic *A. rhizogenes* transformation of *M. truncatula*

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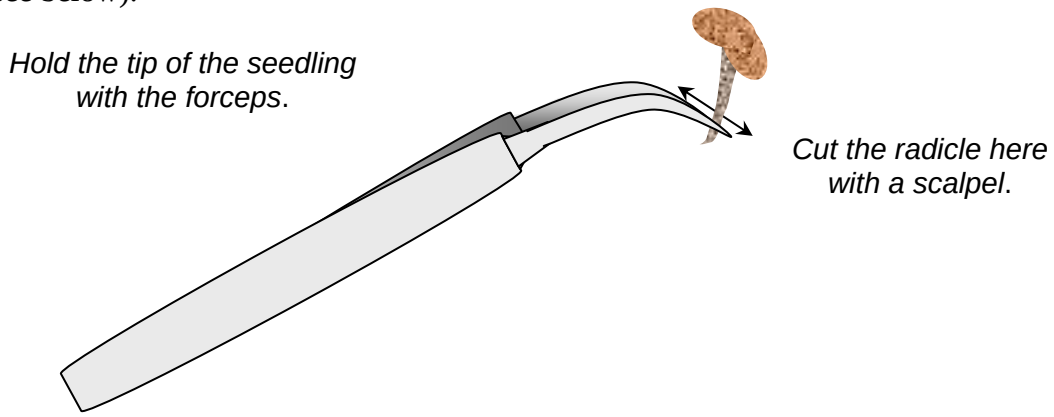
1. Surface sterilize seeds of *M. truncatula* and germinate on inverted agar plates at 14°C.

2. Prepare an overnight liquid culture of *A. rhizogenes* strain ARqua1 (Quandt et al. 1993) containing the binary vector of interest in TY/calcium medium [5g/l Bacto-tryptone, 3g/l Yeast extract, 6mM $CaCl_2$ (added after autoclaving), pH = 7.2, Agar 15g/l] with appropriate antibiotics. 400µl of the bacterial culture is then plated on a TY/calcium agar plate with antibiotics and grow for another 24 h. In this way, a dense bacterial layer is formed which can be used for inoculation of up to 100 plants (communicated by Jean Philippe Combier, LIPM, Castanet-Tolosan)

3. After approximately 30 hr germination, seedlings should have a radicle length of about 1 cm. Under the laminar flow hood, place the seedlings in a glass Petri dish containing water to avoid desiccation of the radicle (not too much water otherwise radicle manipulation is difficult). Cut the radicle approximately 3 mm from the root tip with a sterile scalpel holding the tip with the forceps (see below).



After removing the radicle tip take hold of the seedling by the cotyledons and coat the sectioned surface with *A. rhizogenes* by lightly scraping on the surface of the plate. The seedling is then placed on a Petri dish (approx. 4-6 cm from the top) containing agar with modified Fahraeus medium (see chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions") and Km at 25 mg/l. Up to 10 seedlings can be placed side by side on a single square Petri dish (12 x 12 cm). Seal the dish with parafilm but make several incisions with a scalpel along the upper edge to allow for adequate gas exchange (**very important**). Place the Petri dish at an angle of approx. 45° for 2-3 days (to reduce the risk of the seedlings falling) and then vertically for a further 4-5 days in a 20°C growth room (16-h photoperiod). Co-transformation at 20°C is **twice** as efficient as at 25°C. Note that it is important to include a control without *A. rhizogenes* inoculation to confirm that rooting is inhibited in the presence of kanamycin

4. Approximately 7 days after inoculation transfer the plates to a growth room at 25°C (16-h photoperiod). The first co-transformed roots (i.e. those having integrated both the Ri T-DNA and the binary vector T-DNA) should begin to appear at this stage, with growth clearly initiating from the inoculated radicle section. **50-75%** of inoculated plants should produce transformed roots.

5. About 3 weeks after *A. rhizogenes* inoculation, the transformed roots should be sufficiently well developed for direct experimental studies or excision and clonal propagation (see Chapter "Inoculation and growth with mycorrhizal fungi"). Under optimal conditions an average of **3-4** independent Km-resistant transformed roots can be obtained per inoculated plant (see Boisson-Dernier *et al*. 2001)

Additional general remarks:

(i). Healthy fast-growing transgenic roots are more easily obtained from this radicle sectioning procedure as compared with a classical hypocotyl-wounding approach.

(ii). If it is not possible to use kanamycin selection, transformation can be performed in the absence of direct selection. Under these conditions non-transformed laterals will appear approx. 3-4 days after sectioning, usually emerging from just above the section. Furthermore, in the absence of selection, only a proportion of the Ri-transformed roots (about 60%) will also be co-transformed with the binary vector T-DNA. Whilst Km-resistant selection works well on agar medium, it does not seem to be as effective on certain other solid supports such as phytagel. Aerial plant development of such composite plants is unaffected by the presence of Km in the medium.

(iii). A very useful approach to facilitate the identification of transgenic roots (which can be employed in the absence or in addition to antibiotic selection) is to use a binary vector containing a constitutively-expressed gene encoding a fluorescent marker protein such as DsRED (Limpens et al. 2004). This allows non-destructive and precocious identification of transgenic roots. In the absence of antibiotic selection, it has been reported that up to 50% of transgenic roots can show non-homogeneous expression of the fluorescent marker.

(iv). In general, inoculation of *M. truncatula* with *S. meliloti* directly on the agar medium is not recommended. For nodulating transgenic roots of composite plants they can be transferred to a pouch-paper support (see Boisson Dernier *et al.* 2005; detailed in Chapter "Rhizobial inoculation and nodulation of *Medicago truncatula"*) to aeroponic growth chambers, or to pots (Limpens *et al*. 2004).

B – Protocol for non-axenic *A. rhizogenes* transformation of *M. truncatula*

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Plant preparation:

Following germination, plant the seedlings in soil and place them in the greenhouse for about a month (see Figure 1).

Figure 1: The starting material for composite plant regeneration.

Culture preparation:

1. Grow an overnight culture of *A. rhizogenes* K599 containing the binary vector of interest in YEP medium (with appropriate antibiotic selection). The culture should be grown for at least 16 to 24 h.

2. Pellet the bacteria by spinning at 5,000 x g for 10 min. Pour off the supernatant, and re-suspend the pellet to a final OD_{600} of 0.2 in the Nitrogen-Free Plant Nutrient Medium (no sucrose added).

Plug preparation:

1. Cut "Rock Wool" plugs into sections approximately 0.5 inch thick, discarding the top of the plug. "Rock Wool" is purchased from Hummert International, Earth City, MO, USA (http://www.hummert.com).

2. Place 3 plug sections into a Petri dish lid or bottom (used only for containment) (Fig. 2A).

3. Place the Petri dishes in a tray with a clear, plastic cover.

4. Poke a hole on the top of each plug approximately 3/4 the thickness of the plug using a 200 µL pipette tip. Take care not to punch through the plugs.

5. Inoculate each plug with 5 mL of re-suspended *A. rhizogenes* culture. It is important to add the bacterial suspension with the tip of the pipette and placed it in the poked hole. The bacterial suspension should wet the bottom of the plug first and then move up through the entire plug.

Plant insertion:

1. Cut stem sections approximately 3 cm in length, remove the apical meristem. Each stem section should have one or two axillary buds. Removing the apical meristem reduces the formation of adventitious rooting.

2. Make a slanting cut so that there is more surface area for infection.

3. Insert plant sections into the hole in the plug (Fig. 2B).

4. Replace the cover on the tray and store the cuttings at 25°C for 4-5 days under ambient light.

5. The trays are then opened and the plugs allowed to dry for a few hours, until the leaves are not turgid.

6. After dehydration treatment, plugs are saturated with deionized water and domes/covers returned to the closed position. Plugs are checked periodically and watered when necessary for the remainder of the induction period.

7. The trays are then incubated at room temperature with normal lighting conditions. Approximately two to three weeks later, the first hairy roots should emerge (Fig. 2C).

Figure 2: (A) Explants were inserted into rock wool plugs and placed in a Petri dish. (B and **C**). Hairy roots grow out of the rock wool after 2-3 weeks.

Additional general remarks:

Using the optimised protocol presented here, modified from Collier *et al.* (2005), approx 90% of explants should produce at least 1 transgenic root, with an average of 2-3 transgenic roots per composite plant and 40% of emerging roots transgenic (transgenic roots were scored using a constitutively-expressed fluorescent marker).

Nitrogen-Free Plant Nutrient Medium

Stock	g/100ml	ml stock/ liter PNS
MgSO4.7H2O	6.15	2
CaCl2.2H2O	7.35	4
K2HPO4.3H2O	3.40	1
K2 SO4	5.50	4
FeCl3.6H2O	0.245	2.5
K citrate	0.26	(not needed unless you want it PO4 free)
Micronutrients	1000 x B5	1
Micronutrients		g per 1 liter
H3BO3	Boric acid	0.142
MnSO4·H2O	Manganese sulfate	0.077
ZnSO4·7H2O	Zinc sulfate	0.1725
CuSO4·5H2O	Cupric sulfate	0.037
NaMoO4·2H2O	Sodium molybdate	0.024
CoCl2·6H2O	Cobalt chloride	0.0025
NiSO4	Nickel sulfate	0.001

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