# *Agrobacterium tumefaciens*-mediated transformation and *in vitro* plant regeneration of *M. truncatula*

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**Table of contents**



## 1. Introduction

Successful genetic transformation of *M. truncatula* was reported for the first time just over 10 years ago (Thomas *et al.*, 1992). Conventional *A. tumefaciens* transformation generally comprises an initial co-culture step involving T-DNA transfer to plant cells in tissue explants, followed by the selection and subsequent regeneration of the transformed cells to whole plants. The limiting step in the entire process is generally the *in vitro* regeneration of transformed cells into transgenic plants. Transformation protocols can be classified according to the nature of the regeneration process: either *via* embryogenesis or organogenesis. The most efficient protocols currently available use **embryogenesis,** which exploits the totipotency of plant cells. However, the efficiency of somatic embryogenesis is highly **genotype-dependent**. Two lines of *M. truncatula*, **Jemalong** and **R108**, have been significantly improved for embryogenesis through cycles of *in vitro* culture to increase regeneration efficiency [note that R108 may belong to a different *M. truncatula* subspecies (ssp. *trycicla*) compared to Jemalong (ssp. *truncatula*; Le Signor *et al.* 2005]. The use of these highly embryogenic genotypes (detailed below) is highly recommended for routine transformation of *M. truncatula*.

Derived from the cultivar **Jemalong,** the highly embryogenic genotype **2HA** was selected through one cycle of *in vitro* culture and 3 cycles of recurrent breeding for embryogenesis (Rose *et al.*, 1999; Thomas *et al.*, 1992), and the genotype **M9-10a** was obtained from a single regenerated embryo, and maintained subsequently *in vitro* by vegetative propagation (Santos and Fevereiro, 2002). In the case of the genotype **R108**, a total of 5 cycles of embryogenesis was used to generate the highly embryogenic genotype **R108-1-c3** (Trinh *et al.*, 1998). It should be pointed out that **Jemalong** and **R108** (and their respective derivatives) are phenotypically and genotypically distinct. They have different symbiotic properties, and differ in growth behaviour both *in vitro* and in the greenhouse. The genome of **R108** is smaller than that of Jemalong (Blondon *et al.*, 1994). Whilst crossing between the genotypes (or ssp.) is therefore not straightforward, it is nevertheless feasible and can generate fertile hybrids. Since

**Jemalong** is the genotype that has been chosen for genome sequencing, as well as for mutagenesis and TILLING programs, it is clearly the genotype of choice for projects requiring genetic crossing of transgenic plants with characterized mutants. In addition to the use of adequate highly embryogenic genotypes, the use of hypervirulent strains of *A. tumefaciens* such as AGL1 or EHA105 can also significantly increase overall transformation efficiency (Trinh *et al.*, 1998; Chabaud *et al*., 2003; Araujo *et al.*, 2004). Finally, several agents have been reported for the selection of transformed cells including kanamycin, hygromycin or phosphinothricin. Embryogenesis-mediated regeneration of transformed cells using the optimised conditions described in this chapter should result in between 15-50 % of explants regenerating transgenic plants within 4-5 months of culture.

Direct **organogenesis** based on shoot formation from cotyledonary nodes has been successfully reported for the **Jemalong** genotype **A17** (Trieu *et al.*, 1996), using phosphinothricin as the selective agent. This method is supposed to be genotype-independent but has not yet been reported for other genotypes. Using direct organogenesis, around 3-5 % of explants yield regenerated transgenic plants within 3-6 months of culture.

Before presenting the different protocols, it should also be mentioned here that an attempt to avoid labour-intensive regeneration by means of *A. tumefaciens*-infiltration of *M. truncatula* floral parts or seedlings has been reported (Trieu *et al.*, 2000). Unfortunately, and despite considerable effort, agro-infiltration has not been repeated or extended by this group, nor corroborated by other laboratories.

Efficient transformation protocols now open the way to routine *in planta* analysis of chimeric constructs. *A. tumefaciens*-mediated transformation generates simple, well characterized insertion profiles consisting of a small number of T-DNA copies (Tinland, 1996). In most cases, stable homozygous transgenic lines can be readily obtained for a given insert in the S2 generation (second generation following self-pollination). In this way, the expression of transgenes of interest (e.g. promoter/gene reporter fusions or over-expression/gene silencing constructs) can be studied in detail at any tissue/stage of plant growth and development. Transgenic lines can be maintained by selfing and if necessary crossed into other genetic backgrounds. Besides transgene expression experiments, T-DNA or transposons (and in particular retrotransposons, which can generate high copy numbers of insertions per plant; see Chapter "Insertional mutagenesis in *M. truncatula* using *Tnt1* retrotransposon"), can be used to inactivate plant genes, and thereby provide a convenient tag to identify the inactivated gene of interest (Scholte *et al.* 2002; d'Erfurth *et al.* 2003).

The following four protocols are presented in this chapter, depending on the regeneration process used (**embryogenesis** or **organogenesis**) and on the genotype (**Jemalong** or **R108**):

**A** - *Transformation and regeneration via somatic embryogenesis:*

- **A1** - **Jemalong**, genotype **2HA**.
- **A2** - **Jemalong**, genotype **M9-10a**.
- **A3** - **R108-1c3**.

**B** - *Transformation and regeneration via organogenesis of* **Jemalong**, genotype **A17**.

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# A1 – Transformation and regeneration of Jemalong 2HA via somatic embryogenesis.

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**Introductory comments**:

Leaflets from axenic 2HA plantlets grown *in vitro* are wounded and co-cultivated with the hypervirulent *A. tumefaciens* strain AGL1. Co-cultivation (3 days long) is performed with continuous kanamycin selection (the binary vector carries the *nptII* gene under the control of the *nos* promoter). Explants are then decontaminated by extensive washing in liquid medium in the presence of antibiotics. Subsequent regeneration of transformed plants involves callogenesis followed by somatic embryogenesis. Under these optimised conditions, approx. 25 % of the 2HA explants should give rise to transgenic Km-resistant plants within 4-5 months**.**

**References:**

**- The transformation/regeneration protocol:** Chabaud *et al.* (1996) Plant Cell Reports 15:305-310. Chabaud *et al.* (2003) Plant Cell Reports 22: 46-51.

**- 2HA:** genotype 2HA3-9-10-3: Nolan *et al.*(1989) Plant Cell Reports 8:278-281. Seed requests to Ray Rose: birjr@alinga.newcastle.edu.au or Mireille Chabaud: mchabaud@toulouse.inra.fr

**- AGL1:** Lazo *et al.* (1991) Biotechnology 9: 963-967.

**Detailed Protocol**

***3 weeks prior to transformation* - *Preparation of explants:***

**- Seed sterilization and germination:** see Chapter “Seed storage and germination”.

**- Plantlet growth :** germinated seeds (with radicules 0.5 to 1 cm long) are planted in Magenta boxes (4 plantlets/ Magenta box) containing ≅ 60 ml SHb10 medium, and incubated in a growth chamber at 25°C, with a 16h/8h photoperiod for **2 to 3 weeks**.

**Comments:**

(i). Do not use explants from older plants since this is critical for transformation efficiency.

(ii). 8 Magenta boxes (32 plantlets) will provide a total of approx. 100 leaflets for transformation.

***1-3 days prior to transformation* - *Preparation of the bacterial suspension for co-cultivation:***

1. Streak out AGL1 containing the binary plasmid of interest on TY/Ca medium containing rifampicin (20 mg/l) and ampicillin (50mg/l) and the appropriate antibiotic for plasmid selection (in our case, kanamycin 50 mg/l).

2. 24 h before transformation take a loop of bacteria from the fresh plate and grow overnight in 20 ml of liquid TY/Ca (Rif20/Amp50/Kan50) in a 100 ml Erlenmeyer flask at 28°C (rotary shaker 200 t/mn).

**Comments**

(i). The hypervirulent strain AGL1 is twice as efficient for transformation as LBA4404.

(ii). It is advisable to prepare the transformation medium in advance: for each construct. In order to regenerate ≅ 20 independent transgenic lines, 96 leaflets are co-cultured with 12 leaflets/ Petri dish - hence 8 Petri dishes of solid CIM are required (20 ml/plate). In addition 2 dishes are required for the controls.

***Day1* - *Explant wounding and co-cultivation with Agrobacterium tumefaciens:***

Centrifuge the bacterial culture at 5000g for 10 minutes and re-suspend in 10 ml of sterile deionised water. Dilute to obtain 10 ml of a bacterial solution at $OD_{600}$=0.1.

**The following steps are all performed in a horizontal laminar flow hood.**

1. Pour the bacterial solution into a small sterile glass Petri dish (5.5 cm diameter).

2. Choose **young** healthy well-expanded leaves from the 2-3 week-old *in vitro* plantlets. Transfer 4 trifoliate leaves at a time to a large glass Petri dish (14.4 cm diameter) containing sterile water. After cutting off the three folioles at their base, each foliole is wounded with 3 to 4 scalpel cuts (see Fig. 1). Manipulating the explants in water avoids desiccation. Transfer the wounded explants to the bacterial solution and leave them in the solution for several minutes. Then transfer the leaflets, abaxial side up, to a Petri dish containing solid CIM medium (Callus-Inducing-Medium) + kanamycin 50mg/l (12 folioles/dish). Seal the dishes with parafilm and co-cultivate the leaflets with the bacteria for 3 days in the culture room (25°C, 16h/8h photoperiod).

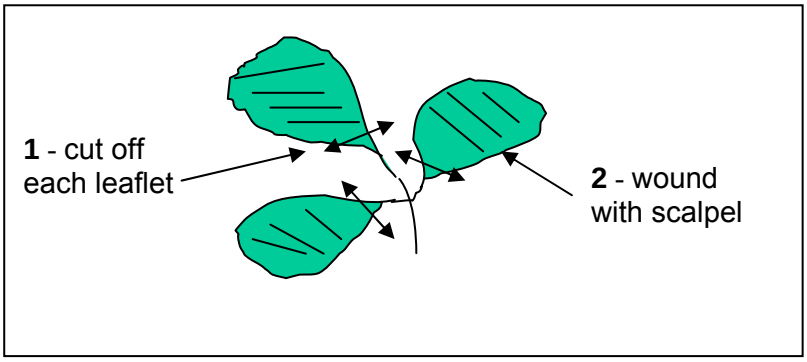


**Figure 1**. Explant wounding.

**Comments:**

(i). As mentioned earlier the choice of the leaflets is very important to insure good transformation efficiency. Only young green expanded leaves should be used (usually a maximum of one to two leaves per plant). The other leaves will be either too old, or insufficiently developed. If the plants are not well developed do not use them!

(ii). For **contro**l experiments **leaflets** are dipped in **water** instead of bacterial solution. In routine experiments, a total of 24 leaflets are wounded and used for controls. 12 are used to test the regeneration media (without kanamycin selection) and 12 are placed on media containing kanamycin to control for efficient selection.

***<u>Day 3</u> - De-contamination of the explants in liquid medium after co-cultivation:***

1. Prepare sterile 100 ml Erlenmeyer flasks containing 20 ml of liquid CIM medium with kanamycin 50mg/l and augmentin 400mg/l (5 parts amoxicillin/1 part clavulanic acid, Beecham Laboratories, France).

2. To eliminate excess bacteria explants are first dipped in sterile water, and then blotted onto sterile filter paper. Excess bacteria should remain stuck to the filter paper.

3. Explants are then transferred to the flasks (12 leaflets/flask)

4. Explant washing is for 3 days with continual agitation (150t/mn) at 25°C, 16h/8h photoperiod.

**Comments:**

(i). The bacterial decontamination step is essential. If this washing step is not performed the bacteria will overgrow the explants often leading to subsequent death of the plant tissue (Chabaud *et al.*, 1996).

(ii). AGL1 is resistant to ampicillin, so carbenicillin cannot be used to decontaminate the explants following co-cultivation. For this reason the antibiotic mix augmentin is used.

***<u>Day 6</u> - Transfer of explants to callogenesis-inducing medium:***

1. After washing, the entire content of each Erlenmeyer flask (liquid medium + leaflets) is poured into a sterile glass Petri dish and the leaflets transferred abaxial side up to a Petri dish containing 20 ml of solid CIM medium including kanamycin (50mg/l) and augmentin (400 mg/l) for callogenesis (12 leaflets/dish).

2. Every 3 weeks, explants are subcultured on fresh CIM medium containing the same antibiotics.

**Comments:**

(i). The leaflets can be rapidly blotted onto sterile filter paper before transfer to CIM medium but this is not essential.

(ii). The first kanamycin-resistant calli are generally seen within 3 weeks of culture on CIM. They appear as green spots on the pale-brown non-transformed tissue of the leaflets. Within 1 month of culture approximately 40% of the leaflets should develop Km-resistant calli. This reaches 90% after 2.5 months, and no new calli are observed after 3 months. After 1 to 2 weeks the green calli turn brown, as they become embryogenic.

***<u>4-6 weeks onwards</u> - Induction of somatic embryogenesis:***

Once a week, calli which are sufficiently well-developed (around 1cm diameter) are transferred for embryogenesis to solid EIM medium (Embryo-Inducing-Medium) including kanamycin (50 mg/l) and augmentin (**200mg/l**). Each callus is transferred independently to a small Petri dish (5.5 cm diameter) containing 9 ml medium. Embryogenic calli are then subcultured every 3 weeks on the same medium. The separation of individual calli is useful to ensure that only one plant is regenerated per callus, and hence that all regenerated plants derive from independent transformation events. This precedure also limits loss of material if there is fungal contamination in a single petri dish.

**Comments:**

(i). Embryogenesis is improved on EIM medium but it is not rare to see embryos already on CIM medium. Embryos are very easily distinguished from the brown callus because they are green and with a round smooth shape.

***Embryo development to plantlets:***

Once a week, embryos which have developed on calli (from globular to torpedo stages), are removed and transferred for further development to EDM medium (Embryo-Development-Medium) including kanamycin (50mg/l) and augmentin (200 mg/l).

**Comments:**

(i). This step can be very long. Some embryos go through secondary embryogenesis instead of developing into a plantlet. Secondary embryos have to be transferred every 3 weeks onto fresh EDM medium until a few of them develop shoots. In a few cases, embryos develop directly to an entire plantlet with shoot and root (these are transferred directly to PDM medium). Leaf development is indicated by the presence of trichomes (these are not present on embryos).

***Rooting of shoots:***

As soon as leaves are visible, leafy stems can be transferred for rooting to a Petri dish containing PDM medium (Plant Development Medium) **without** antibiotics (neither kanamycin nor augmentin). When plantlets are completely developed (with shoot and root), they are then transferred to a Magenta box (1 plant/box) containing PDM. Transgenic plants are maintained *in vitro* by taking cuttings every 2 months. Cuttings are made by transferring the apical node (about 2 cm of stem with a developed leaflet and a bud) to a new Magenta box. The node is placed horizontally onto (and **not** pushed into) the medium to insure efficient rooting. Once a root has initiated from the cutting, one or two weeks later, make sure that it contacts the medium to avoid drying.

**Comments**

(i). In most cases when several kanamycin resistant plants are regenerated from the same kanamycin-resistant callus, they present the same transformation profiles as observed by southern blotting.

(ii). Within 4 to 5 months of culture, 25 % of explants give rise to entire kanamycin resistant plantlets. More transgenic regenerants can be obtained if necessary by extending the tissue culture period. For each construct, 15-20 independent transgenic regenerants are generally analysed. About half of the transgenic plants contain only one copy of the transgene. The selected primary regenerant lines are then grown for seed production and homozygous transgenic lines can be selected in the selfed progeny.

***Acclimatization of* in vitro *regenerated transgenic plants to the green house:***

For green house acclimatization, it is very important to use well-developed plants with a good quality root system (white, thin roots). These are best obtained by taking cuttings from initial regenerants. Avoid any plants with thick vitrous roots, as these are fragile, they will probably not survive acclimatization. 1-1½ month-old plantlets are transferred to a 50/50 sand/soil mixture which has been extensively watered, and each plant is covered with an upside-down Magenta box for a few days. The Magenta box cover is then progressively lifted up over the following few days before being completely removed when the plantlet has acclimatized to the drier greenhouse environment. Acclimatization is preferentially performed with low light

to avoid rapid dessication of the plantlets (pots can be placed on the floor for example, under the shelves).

**Comments concerning the control experiments**

(i). Leaflets dipped in water and cultured on CIM (with kanamycin) shouldn't develop any calli even if they swell slightly.

(ii). Leaflets dipped in water and cultured on CIM (without kanamycin) should regenerate into plantlets more rapidly than the kanamycin-resistant calli. In 3 weeks, calli should already be well-developed, and are often embryogenic on CIM. This control is useful to get used to the regeneration process (in the absence of kanamycin selection), and regenerated (non-transformed) plantlets can be obtained within only 1.5 months.

**Media:**

Optimised growth and regeneration media for *Medicago truncatula* cultivar Jemalong:

- SHb10: Shenk and Hildebrandt for plant growth (the same as PDM except lacking AIB and with agar instead of Phytagel)
- CIM: Callus-Inducing Medium: (Uchimiya and Murashige based-medium)
- EIM: Embryo-Inducing Medium:(Uchimiya and Murashige based-medium)
- EDM: Embryo-Development Medium: (P4 based-medium, Dr Rose, Australia)
- PDM: Plant-Development Medium: (Shenk and Hildebrandt based-medium)

The following are the final compositions of the various media, which are prepared from the stock solutions listed further below:

| | **CIM** | **EIM** | **EDM** | **PDM** |
|---|---|---|---|---|
| | mg/l | mg/l | mg/l | mg/l |
| **Macroelements** | **UM** | **UM** | **P4** | **SH** |
| $KNO_3$ | 1900 | 1900 | 1875 | 2500 |
| $NH_4NO_3$ | 1650 | 1650 | 600 | - |
| $NH_4H_2PO_4$ | - | - | - | 300 |
| $MgSO_4$, $7H_2O$ | 370 | 370 | 225 | 400 |
| $MnSO_4$, $H_2O$ | 16.9 | 16.9 | 10 | 10 |
| $ZnSO_4$, $7H_2O$ | 8.6 | 8.6 | 2 | 1 |
| $CaCl_2$, $2H_2O$ | 440 | 440 | 300 | 200 |
| $KH_2PO_4$ | 170 | 170 | 131 | - |
| $H_3BO_3$ | 6.2 | 6.2 | 3 | 5 |
| KCl | - | - | 225 | - |
| **Microelements** | **UM** | **UM** | **P4** | **SH** |
| $CuSO_4$,$5H_2O$ | 0.025 | 0.025 | 0.025 | 0.2 |
| KI | 0.830 | 0.830 | 0.750 | 1 |
| $CoCl_2$, $6H_2O$ | 0.025 | 0.025 | 0.025 | 0.1 |
| $Na_2MoO_4$, $2H_2O$ | 0.250 | 0.250 | 0.250 | 0.1 |
| **Fe EDTA** | | | | |
| $FeSO_4$, $7H_2O$ | 27.85 | 27.85 | 9.19 | 15 |
| $Na_2$ EDTA | 37.25 | 37.25 | 12.29 | 20 |
| **Vitamins** | **UM** | **UM** | **P4** | **SH** |
| Nicotinic acid | 5 | 5 | 1 | 5 |
| Pyridoxin HCl | 10 | 10 | 1 | 0.5 |
| Thiamin HCl | 10 | 10 | 10 | 5 |
| Glycin | 2 | 2 | - | - |
| Myo inositol | 100 | 100 | - | 1000 |
| **Miscellaneous** | | | | |
| Casein Hydrolysate Acid | 2000 | 2000 | 250 | - |
| Bacto-tryptone | 250 | - | - | - |
| MES | 3mM | 3mM | - | - |
| **Growth hormones** | | | | |
| 2,4-D | 1 | - | - | - |
| trans-zeatin | 2 | 1 | - | - |
| I.A.B. | - | - | - | 0.2 |
| | g/l | g/l | g/l | g/l |
| **Sucrose** | 30 | 30 | 30 | 10 |
| **Agar-agar** | 6 | 6 | - | - |
| **Phytagel** | - | - | 2 | 2 |
| **pH** | 5.8 | 5.8 | 5.8 | 5.8 |

Comment: All the media are prepared from stock solutions of macroelements, microelements and vitamins. These stock solutions are stored at -20°C in 50 ml aliquots. The growth regulators, trans-zeatin and IAB, and the antibiotics, kanamycin and augmentin, are added after autoclaving. The pH is adjusted to 5.8 before autoclaving with 1M KOH.

**Stock solutions :**

**Macro elements : 20 X**

For 500 ml :

| Macro | **UM** | **P4** | **SH** |
|---|---|---|---|
| $KNO_3$ | 19 g | 18.75 g | 25 g |
| $NH_4NO_3$ | 16.5 g | 6 g | |
| $MgSO_4,7H_2O$ | 3.7 g | 2.25 g | 4 g |
| $KH_2\ PO_4$ | 1.7 g | 1.31 g | - |
| $CaCl_2,\ 2H_2O$ | 4.4 g | 3 g | 2 g |
| KCl | - | 2.25 g | - |
| $NH_4,\ H_2PO_4$ | - | - | 3 g |

**Microelements : 100 X**

For 500 ml :

| Micro | **UM** | **P4** | **SH** |
|---|---|---|---|
| $MnSO_4,\ H_20$ | 680 mg | 500 mg | 500 mg |
| $ZnSO_4,\ 7H_2O$ | 430 mg | 100 mg | 50 mg |
| KI | 41.5 mg | 37.5 mg | 50 mg |
| $H_3BO_3$ | 310 mg | 150 mg | 250 mg |
| $Na_2MoO_4,\ 2H_2O$ | 12.5 mg | 12.5 mg | 5 mg |
| $CuSO_4,\ 5H_2O$ | 1.25 mg | 1.25 mg | 10 mg |
| $CoCl_2,\ 6H_2O$ | 1.25 mg | 1.25 mg | 5 mg |

**Vitamins : 100 X**

For 500 ml :

| Vitamins | **UM** | **P4** | **SH** |
|---|---|---|---|
| Nicotinic acid | 250 mg | 50 mg | 250 mg |
| pyridoxin | 500 mg | 50 mg | 25 mg |
| thiamin | 500 mg | 500 mg | 250 mg |
| glycine | 100 mg | - | - |
| Myo-inositol | 5 g | 5 g | |

**Other stock solutions:**

**- MES 1M (pH 5.8)**: Dissolve 29.3g MES in 100ml water. Adjust pH with KOH 10 M. Add water to a final volume of 150 ml. Autoclave and stock at 4 °C.

**- FeEDTA (100 X)**: Dissolve separately 1.4 g $FeSO_4$, $7H_20$ in 100 ml and 2.1 g $Na_2$ EDTA, $2H_2O$ in 100 ml. Mix and make up to 500 ml water. Stock at –20°C in 50 ml aliquots.

**- 2-4D (2-4 dichlorophenoxyacetic acid) (0.4 mg/ml):** Dissolve 40 mg in 5 ml EtOH. Make up to 100 ml with water. Store at 4°C. (Add before autoclaving).

**- Trans-zeatin** (**0.4 mg/ml):** Dissolve 40 mg in 5 ml 1M KOH. Make up to 100 ml in water. Filter sterilise and store at 4°C for 3 months maximum. (Add after autoclaving)

**- IAB (indol butyric acid) (0.4 mg/ml):** Dissolve 40 mg in 5 ml 1M KOH and make up to 100 ml with water. Filter-sterilise and store in dark at 4°C. (Add after autoclaving)

**- Kanamycin** (**50 mg/ml)**: **1000 X** stock solution, filter-sterilized, 1 ml aliquots stored at –20°C. (Add after autoclaving)

**- Augmentin** is weighed immediately before use and poured directly into cooled medium after autoclaving. (The stock solution in water is not stable). Reference: 1g amoxicillin/ 200 mg clavulanic acid, I.V. injectable, SmithKline Beecham Laboratoires Pharmaceutiques, Nanterre, France (usually available in your local chemist).

**Preparation of media from stock solutions :**

| For 1 L | CIM | | EIM | | EDM | | PDM | | SHb10 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Macroelements 20 X | UM | 50 ml | UM | 50 ml | P4 | 50 ml | SH | 50 ml | SH | 50 ml |
| microelements 100 X | UM | 10 ml | UM | 10 ml | P4 | 10 ml | SH | 10 ml | SH | 10 ml |
| vitamins 100 X | UM | 10 ml | UM | 10 ml | P4 | 10 ml | SH | 10 ml | SH | 10 ml |
| FeEDTA stock solution | | 10 ml | | 10 ml | | 3.3 ml | | 5.5 ml | | 5.5 ml |
| myo inositol | | - | | - | | | | 1 g | | 1 g |
| casein acid hydrolysat | | 2 g | | 2 g | | 250 mg | | | | |
| bacto-trypton | | 250 mg | | - | | | | | | |
| 2-4 D stock | | 2,5 ml | | - | | | | | | |
| sucrose | | 30 g | | 30 g | | 30 g | | 10 g | | 10 g |
| MES 1M pH 5.8 | | 3 ml | | 3 ml | | | | | | |
| pH | | 5.8 | | 5.8 | | 5.8 | | 5.8 | | 5.8 |
| agar-agar | | 6 g | | 6 g | | - | | - | | 6 g |
| phytagel | | - | | - | | 2 g | | 2 g | | - |
| | | | | | | | | | | |
| added after autoclaving | | | | | | | | | | |
| zeatin stock | | 5 ml | | 2.5 ml | | - | | - | | - |
| IAB stock | | - | | - | | - | | 0.5 ml | | - |

Comment: Media containing Phytagel as the gelling agent must be poured just after autoclaving. This medium cannot be re-melted after it has solidified.

**TY/Ca medium for growth of Agrobacterium:**

Bacto-tryptone 5g/l
Yeast extract 3g/l
$CaCl_2$ 6mM (added after autoclaving)
pH =7.2
Agar 15g/l

**Specific materials:**

- Centrifuge for preparation of the *Agrobacterium* suspension.
- Horizontal laminar flow hood.
- Culture room at 25°C with a 16h/8h photoperiod.
- Shaker for 100ml Erlenmyer flasks (at least 10 flasks).
- Scalpel and fine forceps.
- Small (5.5 cm ) and large (14.4 cm ) sterile glass Petri dishes.
- Sterile filter papers for blotting the explants.
- Sterile Erlenmeyer flasks (100ml) with cotton seals.
- Small (5.5 cm) and standard (9 cm)) sterile plastic Petri dishes.
- Sterile Magenta boxes.

# A2 - Transformation and regeneration through embryogenesis using the Jemalong genotype M9-10a.

***Developed by***:

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## Introductory comments:

An efficient protocol for *Agrobacterium*-mediated transformation and regeneration *via* somatic embryogenesis has been established for the highly embryogenic M9-10a line of *Medicago truncatula* Gaertn. cv. Jemalong. Using M9-10A and the methodology described below, it is possible to recover transgenic plants after 4 months of *in vitro* culture (Araújo et al., 2004). The protocol uses the *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) and carbenicillin for the elimination of bacteria from regenerating explants. Tight selection using 170 μM (100 mg $l^{-1}$) kanamycin reduces "escapes" - false kanamycin-resistant embryos (Duque et al., 2004). The transfer of late-torpedo/dicotyledonary embryos to an agar-solidified medium with a low concentration of carbenicillin increases embryo conversion. These features are essential to make this method less laborious and more efficient, and especially to avoid analysing non-transgenic "escapes".

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Araújo S.S., Duque A.S.R.L.A, Santos D.M.M.F and Fevereiro M.P.S. (2004) An efficient transformation method to regenerate a high number of transgenic plants using a new embryogenic line of *Medicago truncatula cv*. Jemalong. *Plant Cell Tiss. Organ Cult.* **78**:123-131.

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## Detailed Protocol:

### *Plant material*

Plants of the M9-10a line are maintained and micropropagated in a growth-regulator-free medium MS0A [MS0A= MS (Murashige and Skoog, 1962) basal salts and vitamins, 3 %

(w/v) sucrose, 0.7% (w/v) agar] using sub-culture of 2-5 cm stem segments every 30-40 days as described in Neves *et al*. (2001). Young fully expanded leaves from 30 day-old *in vitro* grown plants are used as explants for transformation experiments.

***Culture media and in vitro culture conditions***

Somatic embryogenesis is induced on an Embryo Induction Medium [EIM – MS (Murashige and Skoog, 1962) basal salts and vitamins, 3 % (w/v) sucrose, 0.45 µM (0,1 $mg.l^{-1}$) 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.91 µM (0.2 $mg.l^{-1}$) Zeatin, 0.2 (w/v) Gelrite]. Embryo development/proliferation and maturation uses an Embryo Proliferation Medium [EPM – identical to EIM but without growth regulators]. Somatic embryos are isolated on an Embryo Conversion Medium [ECM=MS0A, MS basal salts and vitamins, 3 % (w/v) sucrose, 0.7% (w/v) Microagar]. Gelrite, Microagar, Zeatin and antibiotics are supplied by Duchefa, the Netherlands. The 2,4-D is supplied by Sigma, USA. The pH of all media is adjusted to 5.8 before autoclaving (121ºC, 20 min). Growth regulators are filter sterilized through 0.2 µm Whatman filters and added to autoclaved (cooled to 40ºC) media. For embryo development, plant regeneration and acclimatization, cultures are grown with a 16 hr photoperiod of 100 $\mu mol.m^{-2}.s^{-1}$ (cool white fluorescent light), a day/night temperature of 24º/22ºC and a relative humidity of 50 % (Phytotron EDPA 700, Aralab, Portugal).

***Preparation of Agrobacterium tumefaciens for plant transformation***

A single colony of the EHA105 strain of *Agrobacterium* harbouring the appropriate $T_i$ plasmids is used to inoculate 5 ml of Luria Broth (LB) medium supplemented with appropriate antibiotics. After overnight incubation at 28ºC, 200 rpm (Innova 2100, New Brunswick Scientific, UK), the bacterial culture is diluted 1:100 in LB medium (with antibiotics) and incubated overnight at 28ºC, 200 rpm. On the day of transformation, this cell culture is centrifuged for 10 min at 3500 rpm (Beckman GS-6R, USA) and the bacterial pellet is re-suspended at an $A_{600nm}$= 1.5 - 1.6 in liquid EIM supplemented with 100 µM (196,2 $mg.l^{-1}$) acetosyringone (Sigma, USA). Bacterial cells are incubated during 30 minutes in the dark to activate the *Agrobacterium* virulence mechanisms.

***<u>Coupled transformation/regeneration procedure</u>***

***Infection and co-culture***

Leaves of *in vitro* grown M9-10a plants are used as explants for transformation. Individual leaflets are placed onto a wet sterile filter paper in a Petri-dish to prevent desiccation and wounded perpendicularly to the midrib using a scalpel blade previously dipped into the *Agrobacterium* suspension. Explants are transferred with the abaxial side-down (7-9 leaflets per Petri dish). Co-culture is carried out on solid EIM supplemented with 100 µM acetosyringone, in a dark growth chamber (Heraeus, Germany) at 23ºC during 5 days.

***Embryogenic calli proliferation and selection of transgenic tissues***

Selection is initiated immediately after the co-culture period. Infected explants are transferred to EIM containing 170 µM (100 $mg.l^{-1}$) kanamycin and 1.2 mM (500 $mg.l^{-1}$) carbenicillin. Cultures are transferred to normal photoperiod conditions. To maintain the selective pressure, embryogenic calli are transferred weekly to fresh selective medium. Three weeks (21 days) after infection, embryogenic calli are transferred to growth-regulator-free EPM maintaining the same selective pressure. Calli are cultivated in this medium until somatic embryos reach the late torpedo/dicotyledonar stage.

***Embryo conversion and plant regeneration***

Somatic embryos at late torpedo/dicotyledonary stages are transferred to ECM supplemented with 170 µM kanamycin and 0.6 mM (250 $mg.l^{-1}$) carbenicillin. About 40 to 50 non-injured

embryos are placed on each Petri dish, with the shoot pole upwards. Embryos are maintained in the light/temperature/humidity conditions described above. Every two weeks, green somatic embryos resistant to kanamycin are transferred to fresh selective ECM until conversion. When plantlets developed roots, carbenicillin is eliminated from the medium and kanamycin concentration reduced to 85 μM (50 $mg.l^{-1}$). Plants with good root systems in kanamycin containing medium are selected as putative transgenic plants ($T_0$ generation). Prior to transfer to the greenhouse, well rooted $T_0$ plants are potted in a mixture of 2:1 soil-vermiculite and covered with a plastic film for acclimatization in a growth chamber. After 1-2 weeks, the plants can be transferred to pots containing soil and grown in the greenhouse.

**Additional comments:**

***Explant infection and co-culture***

Co-culture is performed in the dark to avoid bacterial overgrowth that often leads to explant death. After this co-culture period it is possible that the infected explants becomes yellow. This aspect does not compromise the efficient regeneration of the explants. Using a stereomicroscope (Leica Wild MZ8, Germany) it is possible to observe that the region near wounded tissues remains green.

***Embryogenic calli proliferation and selection of transgenic tissues***

The strong Km selection applied does not affect the callogenic capacity of the explants. Abundant callus development is observed at the edges of the wounded tissues 7-10 days after somatic embryogenesis initiation. Green pro-embryogenic tissues are observed after 15 to 21 days in selective ECM. Generally, almost 100% of the explants develop embryogenic calli, from which 25-45% produce Km-resistant embryos. Km-resistant embryos start to appear a month after initiating selection. Embryo development is asynchronous and it is possible to observe embryo clumps with embryos at different developmental stages.

***Embryo conversion and plant regeneration***

Green somatic embryos in late-torpedo/dicotyledonary stages are isolated from their original calli and transferred to fresh ECM until conversion to plantlets. This operation is carried out using a stereomicroscope which is placed in a horizontal laminar air flow cabinet previously disinfected (sprayed) with commercial ethanol. Somatic embryos are very vulnerable structures and can be easily damaged during manipulation. In such situations, it is common to observe oxidation around and on the isolated embryo. Embryo-to-plant conversion is improved by reducing secondary embryogenesis and hyperhydricity transferring late-torpedo/dicotyledonary embryos to agar-solidified medium with half of the initial concentration of carbenicillin. Transfer to soil is not a limiting step when plants with *in vitro* well-developed root system are chosen.

***Important features of this protocol***

(a). Use of the highly embryogenic M9-10a line: 97-100 % of leaflet explants produce embryogenic calli even under selection. During the regeneration process no special *in vitro* culture requirements, media or supplements are necessary. Growth regulators are used in low concentrations and only during the first 21 days of the induction of somatic embryogenesis, reducing the possible occurrence of somaclonal variation.

b). Use of strong selection (100 mg/l kanamycin) makes the transformation system efficient since only NPTII transformed embryos/plants are recovered, reducing wasted time analyzing "escapes" .

c). Recovery of several independent *M. truncatula* cv. Jemalong transgenic lines in a short period of time (4 months).

# Media

| | MS0A = ECM | EIM | EPM |
|---|---|---|---|
| Basal Medium | MS Major and Minor Salts | MS Major and Minor Salts | MS Major and Minor Salts |
| Vitamins | MS Vitamins | MS Vitamins | MS Vitamins |
| Carbon Source | 3% Sucrose | 3% Sucrose | 3% Sucrose |
| Solidifying agent | 0.7 % (w/v) Microagar | 0.2 % (w/v) Gelrite | 0.2 % (w/v) Gelrite |
| Growth regulators | no | 0.45 μM 2,4 -D<br>0.91 μM Zeatin | no |

EIM = Embryo Inducing Medium; EPM = Embryo Proliferation Medium; ECM = Embryo Conversion Medium

## Equipment required

Laminar flow cabinet, dark growth chamber, plant growth chamber, forceps, scalpel, blades, sterile tips, micropipets, filter paper, Petri dishes, Erlenmeyer flasks 100 ml, Falcon tubes (15 ml and 50 ml), orbital shaker, stereomicroscope.

## Schematic representation of the M9-10a transformation procedure

| **Plant material** | **Explant infection and co-culture** | | **Somatic embryogenesis induction** | | **Embryo maturation and proliferation** | | **Embryo conversion** | | **Plantlet rooting** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Med.*** | ***Suppl.*** | ***Med.*** | ***Suppl.*** | ***Med.*** | ***Suppl.*** | ***Med.*** | ***Suppl.*** | ***Med.*** | ***Suppl.*** |
| Wounded leaflet explants of M9-10a | EIM | 100 μM aceto-syringone | EIM | 100 mg/l Km<br>500 mg/l Carb | EPM | 100 mg/l Km<br>500 mg/l Carb | ECM | 100 mg/l Km<br>250 mg/l Carb | ECM | 50 mg/l Km |
| | 5 days | | 3 sub-cultures (16 d) | | 4-8 sub-cultures (30-60 d) | | 2-4 sub-cultures (15-30 d) | | 2 sub-cultures (15 d) | |

EIM = Embryo Inducing Medium; EPM = Embryo Proliferation Medium; ECM = Embryo Conversion Medium

# A3 - Transformation and regeneration of R108-1(c3) via somatic embryogenesis

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**Introductory comments:**

Previous versions of the protocol described below were published in Trinh *et al.* (1998) and Kamaté *et al.* (2000), and the most recent in Cosson *et al.* (2006).

Using the transformation protocol described here for genotype R108-1 (c3), from 50 to 80% (depending on the selection marker used) of the original explants produce transgenic embryogenic calli that can subsequently regenerate numerous transgenic plants. Thus, transformation efficiency can be as high as 50 independent transgenic plants from 100 infected leaf explants.

Selection for Basta or hygromycin is particularly convenient for R108. Vectors conferring kanamycin resistance should be avoided because selection for this antibiotic is not so stringent and often results in the regeneration of escapes (i.e. plants which are not transformed; d'Erfurth *et al.* 2003). We have also observed that pBin19-derived vectors (Bevan, 1984) give complex T-DNA integration patterns corresponding to the formation of concatamers (Scholte *et al.* 2002). These vectors should be avoided in order to simplify the analysis of the transgenic plants.

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## Detailed Protocol:

### *Preparation of plant material for transformation:*

The growth cycle of R108 takes approx. 6 months from seed to seed. Either *in vitro* plants or greenhouse-grown plants can be used for transformation experiments. Growth conditions are important for the success of transformation/regeneration and unhealthy plants should not be used for these experiments. For example, avoid chemical treatments before the experiment and choose leaves without insect/fungal damage in the case of greenhouse plants.

### *Preparation of plant material from sterilized seeds:*

1. Seeds are scarified with sand paper, sterilized for 30 min in a Bayrochlor solution, rinsed 4 times in sterile water and germinated on sterile wet Whatmann 3MM paper in 9 mm diameter petri dishes, in the dark at room temperature.

2. After two days seedlings are transferred into half-strength SH9 medium in 1 litre glass pots (or magenta boxes) and grown for 3 weeks in the growth chamber (24°C; 16H light) before leaf explants can be used for transformation.

### *Preparation of plant material from greenhouse-grown plants:*

1. Seed are scarified with sand paper and germinated in sand for two weeks in the greenhouse (sixteen-hour day period, 60% relative humidity, 22°C day/16°C night temperature with additional light: 200 $\mu E/m^2/s$). At this stage, seeds are sown at high density (1 plant per $cm^2$) in a 10 cm deep tray and watered until they are fully germinated. During this time the tray is covered with a transparent plastic cover or with plastic sheet. Once the first leaf appears the plantlets are watered with nutrient solution and the tray can be opened.

2. Two-week-old plantlets with 2-3 leaves are then transferred to plastic pots with vermiculite or a mixture of soil and sand (3:1) in the greenhouse (16 hour day period, 60% relative humidity, 22°C day/16°C night with additional light: 200 $\mu E/m^2/s$) or in growth chambers (16 hour day period, 60% relative humidity, 22°C with 200 $\mu E/m^2/s$ light) for three weeks before the transformation experiment.

3. Plants are watered twice a day alternatively with nutrient solution and water. Watering should be carefully controlled in order to avoid excess humidity. Ideally, the soil surface should start to dry between each watering. If the surface remains humid in order to reduce pathogen attack it is necessary to reduce the frequency of watering.

4. Three weeks later, plants (15 cm high, 10-20 expanded leaves) should be ready for *in vitro* transformation experiments.

5. Young expanded leaves are used for the transformation experiments. These leaves are generally robust enough for the sterilization step.

6. Alternatively, entire flowers of older plants can be used (Kamaté *et al.* 2000). In this case the petals, sepals and pistils of young open flowers are used for the transformation experiment. This makes it possible to use the greenhouse grown plants for a longer period of time.

***Preparation of Agrobacterium culture:***

1. Two days before the transformation experiment, initiate an *Agrobacterium* liquid culture by inoculating a freshly grown single colony of *Agrobacterium* in 2 ml YEB liquid medium supplemented with the appropriate antibiotics for the selection of the transformation vector. Addition of acetosyringone, generally used to induce T-DNA transfer, is not required for *Medicago truncatula* R108 transformation. Incubate with shaking at 30$^{o}$C (200 rpm) overnight.

2. The day before the transformation, a 300 ml flask containing 30 ml *Agrobacterium* culture (with appropriated antibiotics for selection) is inoculated with 1 ml of the 2 ml overnight pre-culture. This culture is shaker incubated in a 200 mL Erlenmayer flask at 30°C over night (200 rpm). The $OD_{600nm}$ of this culture should reach 0.6 the day of the transformation

3**.** Centrifuge the 30 ml *Agrobacterium* culture at 3000 g for 20 min and resuspend the pellet gently in 50 ml sterile SH3a liquid medium ($OD_{600nm}$= 0.6). The culture is then transferred to a vacuum flask (see below).

***<u>Leaf explant preparation and infiltration:</u>***

For transformation, choose leaves from 4-6 week-old plants either grown in *in vitro* culture or the greenhouse. These leaves should be round, healthy, and without too many hairs. Five leaflets are used per plate. Each leaflet can generate at least one transformed plant. **If you use *in vitro* plants go directly to Step 5.**

1. Sterilization of greenhouse-grown leaves is carried out in a 50 ml falcon tube (20-30 leaves per tube). Leaflets are first rinsed in tap water containing 2-3 drops of teepol. The tubes are inverted several times in order to wet all the leaves, and then they are rinsed with tap water until no more foam is present.

2. Replace water by Na-hypochlorite solution (6° Cl), mix gently and leave the tube for 7 min in a rack (lid up). Invert the tube (lid down, standing on the bench) and wait for an additional 7 min.

3. All manipulations after this step should be done **under sterile conditions**.

4. Rinse the leaflet three times with sterile water in the same 50 ml falcon tube.

5. Place the sterilized leaflets into a 9 cm Petri dish with approximately 30 ml sterile water. Cut the leaflets into square pieces by removing the edges of the leaflets with a sterile scalpel.

6. Place the cut leaf pieces into the *Agrobacterium* culture (prepared as above) in a sterile vacuum flask (20-30 leaf pieces per 50 ml culture). Shake the flask gently to separate the leaf pieces.

7. Apply a vacuum (650 psi) to the leaf explants in the *Agrobacterium* culture for 20 min. In order to avoid cell damage the vacuum should be released slowly. This agrobacteria infiltration step is important for high frequency transformation.

8. Once the vacuum is released, the vacuum flask is placed on a shaking (50-60 rpm) table at room temperature for 1-2 hours to allow the tissue to recover from the infiltration procedure.

***Co-cultivation (48 h):***

This step allows the bacteria in contact with the plant cells to transfer the T-DNA to the plant nucleus. It is during this period that the transformation process occurs.

1. Under the sterile laminar flow, transfer the explants to an empty 9 cm Petri dish and remove as much as possible of the bacterial solution with a pipette. This agrobacteria solution should be sterilized with Na-hypochlorite before discarding.

2. Transfer the leaf explants to solid SH3a medium without antibiotics. The lower side (abaxial) of the leaf explants should be in contact with the medium.

3. The plates are then sealed with alimentary plastic film and incubated for a maximum of 2 days in the dark in the plant growth culture room (24°C). It is important to use the alimentary film rather than parafilm during transformation in order to allow gas exchange between the plant material and the outside atmosphere. During this co-cultivation step care should be taken that the agrobacteria do not over-grow the leaf explants.

***Callus formation with selection (5 to 6 weeks):***

This step will allow the transformed tissue to grow and form calli. In addition, the hormone treatment will induce embryogenesis. By the end of the callus formation step, the pre-embryos should have formed.

1. The leaf explants are removed from the co-cultivation medium and wiped gently on fresh solid (SH3a or SH9) medium in order to remove excess bacteria that have grown on the explants.

2. The leaf explants are transferred to fresh SH3a medium with 800 mg/L Augmentin (to eliminate the agrobacteria) and with 3 mg/L Basta (Glufosinate-ammonium) to select for the transformed cells if the vector confers Basta resistance. If the vector used for transformation confers kanamycin resistance, then 40 mg/mL Km should be used. If the vector confers hygromycin resistance, use 10 mg/L hygromycin B.

3. Plates are sealed with alimentary plastic film (see above) and placed in the dark in the growth chamber (24°C) for 5 to 6 weeks. Check plates regularly for contamination.

4. The herbicide or antibiotic resistant callus material can be seen two weeks after infiltration. These calli are transferred to fresh SH3a medium every 2-3 weeks.

***Embryogenesis (3-6 weeks):***

At this stage the calli look like brown sugar powder and are transferred to hormone-free medium in the light. These two changes induce embryogenesis followed by plantlet development. Selection can be maintained in the SH9 medium for the first three weeks to reduce escapes but should be removed in subsequent sub-cultures because it reduces the regeneration capacity of the plant. If at this stage the agrobacteria start to grow again, the calli should be transferred to SH9 medium containing Augmentin (and Basta if necessary).

1. Transfer calli to SH9 medium and cultivate in the light (130 $\mu E/m^2/s$; in an *in vitro* growth chamber (24°C, 12h photoperiod).

2. Calli are then transferred to fresh SH9 medium every three weeks until the pre-embryos appear (between 3-6 weeks on this medium). Calli at this stage should be friable and start to turn green.

3. From the calli, pre-embryos will develop into true embryos between 20-30 days after transfer to the SH9 medium. Many embryos (from 5-20) should develop from each callus and give rise to transgenic siblings. Thus, in order to obtain independent transgenic plants, we generally keep only a few embryos (5-10) at this stage and a **single transgenic plant per original explant** at the end of the transformation experiment.

***Plantlet development (2 to 6 weeks):***

1. About 2 to 3 weeks later, plantlets start to develop from the embryos.

2. When plantlets are formed transfer them to half-strength SH9 medium to induce rooting.

3. When rooting starts, the plantlets should be transferred to half-strength SH9 square plates (120 x 120 mm) which are placed vertically (or at a 45° slant) in the growth chamber to allow roots to grow along the medium (*M. truncatula* R108 roots grow poorly inside the medium. The amount of Kalys agar should be increased to 9g per litre to produce a more solid medium. Rooting of the plantlets on this medium takes 2 to 6 weeks.

***Transfer of the transgenic plants to the greenhouse:***

The plant material transferred from *in vitro* conditions to the greenhouse is very sensitive to the change in humidity. Thus, to avoid loss, plants should be maintained at the beginning of transfer in water-saturated conditions and then adapted progressively to the normal greenhouse conditions.

1. Plantlets that have developed a few leaves and roots on half-strength SH9 medium are transplanted into a tray containing sterilized (or clean) sand covered with a transparent lid.

2. Plants should be watered with tap water during the first two weeks and subsequently with nutrient solution. A plate is placed under the tray in order to keep it water-saturated.

3. The sand should always be humid. For the first 5 days the lid is kept closed to keep the plants in an atmosphere-saturated with water. Then the lid is progressively opened to slowly reduce the humidity level.

4. At the end of the second week, the lid can be completely remove. The plants are then watered alternatively with nutrient solution and water.

5. When the plants have developed new leaves under the greenhouse conditions, they can be transferred to pots with a mixture (3:1) of soil and sand.

6. These plants are termed T0 plants. If they develop normally they should flower and set seeds after 2 to 3 months.

7. The selection for Basta-resistant transgenic plants grown in the greenhouse can be performed at this stage by spraying a solution of 120 mg/L gluphosinate-ammonium onto the plantlets.

8. Complete development of the plant will take from 4 to 6 months. Approximately 50% of the transferred plantlets will survive the green-house transfer and develop into plants. Sometimes these plants grow poorly and/or produce few seeds. As a consequence seed production of the T0 plants can vary from a few pods to several hundred seeds par plant. Normally this improves in the subsequent generation (T1 plants). If some plants develop leaves that are larger and thicker than usual they may be tetraploids. We have regularly detected a low percentage of such tetraploid plants in our regeneration experiments.

# Media:

| N6 major salts | | |
|---|---|---|
| **Chemicals** | **Amount for 1 L** | **Final concentration in the stock solution** |
| $MgSO_4.7\ H_2O$ (dissolve completely) | 1.85g | 7.5 mM |
| $KNO_3$ | 28.30g | 280 mM |
| $(NH_4)2SO_4$ | 4.63g | 35 mM |
| $CaCl_2.2\ H_2O$ | 1.66g | 11 mM |
| $KH_2PO_4$ | 4.00g | 30 mM |
| $H_2O$ | QSP 1 L | -- |

***Table 1****: Composition of the N6 major salts* (Chu, 1978). *This solution can be stored at 4°C.*

| SH minor salts | | |
|---|---|---|
| **Chemicals** | **Amount for 100 ml** | **Final concentration in the stock solution** |
| $MnSO_4.\ H_2O$ | 1g | 60 mM |
| $H_3BO_3$ | 500mg | 80 mM |
| $ZnSO_4.7\ H_2O$ | 100mg | 3.5 mM |
| KI | 100mg | 6 mM |
| $Na_2MoO_4.2\ H_2O$ | 10mg | 1 mM |
| $CuSO_4.5\ H_2O$ | 20mg ($CuSO_4$=12.8mg) | 0.8 mM |
| $CoCl_2.6\ H_2O$ | 10mg | 0.4 mM |
| $H_2O$ | QSP 100 mL | -- |

***Table 2:*** *Composition of SH minor salts* (Schenk and Hildebrandt, 1972). *This solution can be stored at 4°C without autoclaving.*

| SH vitamins | | |
|---|---|---|
| **Chemicals** | **Amount for 100 ml** | **Final concentration in the stock solution** |
| Nicotinic acid | 500mg | 40 mM |
| Thiamine HCl (B1 vitamin) | 500mg | 15 mM |
| Pyridoxine HCl (B6 vitamin) | 500mg | 24 mM |
| $H_2O$ | QSP 100 mL | -- |

***Table 3:*** *Composition of SH vitamins stock* (Schenk and Hildebrandt, 1972). *This solution can be stored at 4°C without autoclaving.*

| **Chemicals or stocks** | **SH3a** | **SH9** | **½ SH9** |
|---|---|---|---|
| N6 major | 100 mL | 100 mL | 50 mL |
| SH minor | 1 mL | 1 mL | 0.5 mL |
| SH vitamins | 1 mL | 1mL | 0.5 mL |
| EDFS (stock solution) | 20 ml | 20 mL | 10 mL |
| Myo-inositol | 100 mg | 100 mg | 50 mg |
| Sucrose | 30 g | 20 g | 10 g |
| 2-4 D | 4 mg | -- | -- |
| BAP | 0.5 mg | -- | -- |
| pH | 5.8 | 5.8 | 5.8 |
| H2O | QSP 1 L | QSP 1L | QSP 1L |
| Phytagel | 3 g | | |
| Kalys agar | | 7g | 7g * |

***Table 4:*** *Preparation of the SH3a, SH9 and half-strength SH9 media. For each medium the quantities indicated are for preparing 1 liter of medium.* ******* *Use 9 g of kalys agar for vertical or slant plates.*

| Chemicals | SH3a | SH9 | ½ SH9 |
|---|---|---|---|
| $MgSO_4.7\ H_2O$ | 0.75 mM | 0.75 mM | 0.375 mM |
| $KNO_3$ | 28 mM | 28 mM | 14 mM |
| $(NH_4)2SO_4$ | 3.5 mM | 3.5 mM | 1.75 mM |
| $CaCl_2.2\ H_2O$ | 1.1 mM | 1.1 mM | 0.55 mM |
| $KH_2PO_4$ | 3 mM | 3 mM | 1.5 mM |
| $MnSO_4.\ H_2O$ | 6 µM | 6 µM | 3µM |
| $H_3BO_3$ | 80 µM | 80 µM | 40µM |
| $ZnSO_4.7\ H_2O$ | 3.5 µM | 3.5 µM | 1.75 µM |
| KI | 6 µM | 6 µM | 3 µM |
| $Na_2MoO_4.2\ H_2O$ | 1 µM | 1 µM | 0.5 µM |
| $CuSO_4.5\ H_2O$ | 0.8 µM | 0.8 µM | 0.4 µM |
| $CoCl_2.6\ H_2O$ | 0.4 µM | 0.4 µM | 0.2 µM |
| Nicotinic acid | 40 µM | 40 µM | 20 µM |
| Thiamine.HCl (vitamine B1) | 15 µM | 15 µM | 7.5 µM |
| Pyridoxine.HCl (vitamine B6) | 24 µM | 24 µM | 12 µM |
| EDFS (stock solution) | 0.38 mM | 0.38 mM | 0.19 mM |
| Myo-inositol | 0.55 mM | 0.55 mM | 0.275 mM |
| Sucrose | 3 % | 2 % | 1 % |
| 2-4 D | 4 mg/L (16µM ) | -- | -- |
| BAP | 0.5 mg/L (2µM) | -- | -- |
| pH | 5.8 | 5.8 | 5.8 |

***Table 5:*** *Composition of the SH3a, SH9 and half-strength SH9 media.*

### *Gelling agents:*

*M. truncatula* is very sensitive to the type of agar used. Phytagel works well for R108 during the callus-inducing period. For other media we use Kalys agarHP 696. Other gelling agents can induce browning and death of the explants (Kamaté *et al.* 2000)

**Phytagel (**Sigma: P-8169) is used for the SH3a medium.

**Kalys agar** HP 696-7470 (Kalys, 39 Avenue Jean Lebas, 59100 Roubaix, France) is used for the SH9 and ½ SH9 media.

## Plant material:

*M. truncatula* (Gaertn.) line R108-1 (c3) is described in Trinh *et al*. (1998) and called R108 throughout this text. *M. truncatula* R108 seeds for transformation experiments can be requested from P. Ratet (Pascal.Ratet@isv.cnrs-gif.fr).

## Agrobacterium strain, T-DNA vectors and bacterial growth medium:

Various laboratory-disarmed *A. tumefaciens* strains can be used; however strain LBA4404 is particularly inefficient for *M. truncatula* transformation of R108.

1. *Agrobacterium tumefaciens* EHA105 strain (Hellens *et al*. 2000) is recommended for R108 transformation.

2. Conventional binary vectors other than pBin19 (Bevan, 1984) derivatives can be used for *Medicago* transformation. We have observed that pBin19-derived vectors (Bevan, 1984) give complex T-DNA integration patterns corresponding to the formation of concatamers (Scholte *et al.* 2002). These vectors should be avoided in order to simplify the analysis of the

transgenic plants. Plasmids are introduced into the *Agrobacterium* strain by triparental mating as previously described (Ratet *et al*. 1988), by electroporation or by other techniques.

**YEB medium for *Agrobacterium* culture** (Krall *et al.* 2002): For 1L, add 5 g of Bacto beef extract, 1 g of Bacto yeast extract, 5 g of peptone, 5 g of saccharose, 2 ml of magnesium sulfate (1M stock solution), pH: 7.2. For solid medium, add 15 g/L Bacto agar before autoclaving

**Stock solutions:**

***Phytohormone stocks:***

**2,4 D**: Stock solution 1 mg/ml in water: final concentration 4 mg/L.

**BAP**: Stock solution 1 mg/ml. First dissolved in a small volume of 2M NaOH and then diluted to 1 mg/ml with water. Final concentration 0.5 mg/L.

***Antibiotic stocks:***

**Augmentin** (GlaxoSmithKline, 92731 Nanterre Cedex, France): Stock 0.2 g/ml in sterile water. Final concentration for media is 800 mg/L.

**Basta** (gluphosinate-ammonium, Hoechst schering AgrEvo GmbH, Frankfurt/Main): Stock 200 g/l in commercial solution 'Liberty'. Final concentration for media is 3 mg/L.

**Kanamycin** (Sigma: 25389-94-0): Stock 40 mg/mL in sterile water. Final concentration for media is 40 mg/L.

**Hygromycin** (HygroGold$^{TM}$, InvivoGen: www.invivogen.com): Stock 50 mg/ml. Final concentration for media is 10 mg/L.

**Other materials:**

- Sterilized sand.
- Sand paper (commercial fine sand paper for hard materials).
- Bayrochlor solution is an industrial disinfectant (BAYROL GMBH, Germany). The active compound is sodium dichlorisocyanurate. Use a 7g/L solution for seed sterilisation.
- Glass pots for *in vitro* culture (1 liter volume with bright opening) or Magenta boxes.
- Nutrient solution: N/P/K: 18/6/26 (Soluplant, Duclos International, Lunel Viel, France).
- Teepol L (detergent: http://teepol.co.uk/products.html).
- Na-hypochlorite solution (6 or 12° Cl).
- Vacuum flask (250 ml Erlenmayer form filtration flask) and vacuum pump. A water-tap pump or electric pump can be used.
- Alimentary plastic film.
- *In vitro* growth chamber lights: Alternating Mazdafluor Prestiflux-HF Incandia: 4A TF"P"58W/inc and Mazdafluor Blanc Industrie 33 (6J TF58W/BI) tubes can be used to produce a good light spectrum.

# B - Transformation and regeneration of Jemalong A17 through direct shoot organogenesis

***Developed by:***

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## Introductory Comments:

This method is a modification of the protocol published in Trieu et al. (1996). *M. truncatula* genotypes other than A17 have not yet been tested. The *Agrobacterium tumefaciens* strain LBA4404 is used for transformation and either kanamycin or bialaphos used as selectable markers. *A. tumefaciens* strains EHA 101 and 105 are reported to be more efficient in transformation but they are more difficult to eliminate and sometimes cause overgrowth problems.

## References:

Trieu, A.T and Harrison, M.J. (1996) Rapid transformation of *Medicago truncatula:* regeneration via shoot organogenesis. Plant Cell Reports, 16: 6-11.

Chabaud et al. (1996). Plant Cell Rep. **15,** 305-310.

## Detailed Protocol:

**1 week before**: Place *M. truncatula* A17 seeds at 4 °C.

**12 h before**: Streak a fresh plate of Agrobacterium on **YEP** plus antibiotics from frozen glycerol stock and incubate at 28°C for one or two days (depending on the strain). Include a control strain with empty vector.

**Day 1.** Select good undamaged seeds. Soak seeds in conc. $H_2SO_4$ for 10 minutes (in a fume hood). Rinse 3x with distilled water. Treat 15 minutes with 10 % bleach solution plus 0.1 % Tween 20. Rinse 3x with sterile water. Briefly blot dry on sterile Whatman filter paper and spread seeds on M1 medium in 15x100 mm petri-dishes. Incubate in the dark at least 24 hours at 23-25 °C.

**Day 2.** Transfer the dishes to the light and continue incubation in the light (16 h photo period) at 23-25°C for two days.

Inoculate a single colony of Agrobacterium into 2 ml **TY** medium plus antibiotics and incubate at 28 °C on a shaker at 250 rpm until bacterial suspension is relatively dense (1-2 days)

**Day 4.** Add the 2 ml bacterial suspension to 25 ml liquid **TY** plus antibiotics and grow until O.D.600 reaches 0.5 (4-6 hours). Centrifuge at 8000 rpm for 5'at RT. Keep pellet at RT.

### *Preparing and transfecting cotyledonary explants:*

Transfer 60 seedlings (3 days old) to a sterile petri dish. Resuspend bacterial pellet in 25ml **M2A**. Pipette 3 ml of bacterial suspension onto the seedlings. Bisect the seedlings at the

embryonic axis and remove hypocotyls 1-2 mm below the cotyledonary node (at the point where the colour changes from greenish-brown to white). In this way each explant should have one cotyledon and 1-2 mm of the embryonic axis.

***Cocultivation:***

Briefly blot explants dry with sterile filter paper and transfer to **M2A-agar** in 15x100 mm petri-dishes with the adaxial side **face up** (20-25 explants/plate). Make sure the hypocotyl and cotyledonary basal portions **touch** the medium. Maintain on this medium at **18-20°C** for 5 days with a 16 h photo period.
Include about 20 explants for non-transformed controls and as many as possible for empty vector control.

***Regeneration and selection of transgenic roots:***

**Day 9:** Wash the cotyledon explants 3x with sterile water. Transfer them to **M2R** regeneration medium in 20x100 mm petri-dishes (10 cotyledons/plate). Antibiotic selection begins at this point. Incubate at **23-26°C** under a 16 hr photoperiod.

**Day 17:** At this point, green shoots should be growing from the hypocotyl region of the cotyledon and the remaining part of the cotyledon will start to die. Cut and discard the remaining part of the cotyledon (the part without the shoots). This gets rid of excess agrobacteria. Transfer the remaining explant with shoots to fresh **M2R** medium.

**Day 27:** Transfer to fresh **M2R** medium.

**Day 35:** Transfer explants with resistant shoots to **M3** shoot development medium in Magenta boxes.

Using Bialaphos selection, transformed shoots will have a dark green, vitrified appearance, whereas untransformed shoots will turn yellow or light brown in about 10 days. With Kanamycin selection, the transformed shoots will remain green, while untransformed shoots will start to turn white after about 2 weeks.

**2 weeks later:**

Cut and transfer shoots (0.5 cm or taller) to **M4** rooting medium in Magenta boxes. Lay the shoot **at an oblique angle on** the agar. Roots form mainly from the portion of the cut surface that is not in contact with the agar, so do not submerge the cut end of the stem completely in the medium. Transfer the rest of the shoot clump to fresh **M3** (dead shoots can be removed). Transfer the shoot clumps regularly to fresh M3 medium (every 3-4 weeks).

With Bialaphos selection, after 1 or 2 rounds of selection on M3, it is sometimes helpful to transfer to fresh M3 containing no Bialaphos. Bialaphos seems to inhibit shoot expansion and a period without bialaphos can help some clumps to develop shoots.

**10 days after transfer to M4:** Shoots begin to root.

**10-15 days later:** Well-rooted shoots are ready for transferring to the greenhouse.

**10 days later:** Plants should be well established.

**10 days later:** Plants should have enough trifoliates for southern blotting.

# Media:

All media contain PDM salts and vitamins (Chabaud *et al*., 1996.)

## ***Stock solutions:***

| **PDM salts 100X:** | **g / 400 mL** |
|---|---|
| $KNO_3$ | 100 |
| $NH_4H_2PO_4$ | 12 |
| $MgSO_4 .7H_2O$ | 16 |
| $MnSO_4 .H_2O$ | 0.4 |
| $ZnSO_4 .7H_2O$ | 0.04 |
| $H_3BO_3$ | 0.2 |
| $CuSO_4 .5H_2O$ | 0.008 |
| KI | 0.04 |
| $CoCl_2 .6H_2O$ | 0.004 |
| $Na_2MoO_4 .2H_2O$ | 0.004 |

*(Autoclave, store at RT or 4 °C)*

| **PDM iron and vitamins 100X:** | **g / 1L** |
|---|---|
| Nicotinic acid | 0.5 |
| Pyridoxine HCl | 0.05 |
| Thiamine HCl | 0.5 |
| Myo-Inositol | 100 |
| $FeSO_4 .7H_2O$ | 1.5 |
| $Na_2EDTA$ | 2 |

(Filter sterilize, aliquot in 50 mL falcon tubes and store at – 20 °C or for 2 months at 4°C)

**MES 0.5 M:**

MES (2-(-4Morpholino)-Ethane Sulfonic Acid) MW: 195.24
Dissolve 48.81 g MES in 500 ml water.
Autoclave and store at 4 °C. Discard if contaminated.

**BAP 10 mM:**

BAP (6-Benzylaminopurine) MW: 225.3
Dissolve 0.023 g BAP in 250 µl 1M NaOH (heat gently if needed), and then adjust volume to 10 ml with water.
Filter sterilize and store at 4 °C.

**NAA 10 mM:**

NAA (□-Naphthaleneacetic acid) MW: 186.2
Dissolve 0.019 g NAA in 250 µl 1M NaOH (heat gently if needed), and then adjust volume to 10 ml with water. Filter sterilize and store 4 °C.

**IBA 10 mM:**

IBA (Indole-3-Butyric Acid) MW: 203.2
Dissolve 0.020 g IBA in 3 mL 95% ethanol, and then adjust volume to 10 ml with 70 % ethanol.
Filter sterilize and store at – 20 °C.

**Acetosyringone 1 M:**

Acetosyringone (3', 5' -dimethoxy-4'-hydroxy acetophenone) MW: 196.20
Dissolve 20 mg in 125 µl 100 % DMSO.
Filter sterilize. **Prepare fresh**

**Carbenicillin 500 mg/mL:**

Dissolve carbenicillin in water
Filter sterilize and store at – 20 °C.

**Kanamycin 50 mg/mL or Bialaphos 100 mg/mL:**

Dissolve in water
Filter sterilize and store at – 20 °C.

**M1 (germination medium):** For 1L:

| | |
|---|---|
| PDM salts | 10 mL |
| $CaCl_2$ .$2H_2O$ | 0.2 g |
| Sucrose | 10 g |

- Adjust the pH to 5.8 with KOH, then adjust the volume to 990 mL.
- Add Agar-agar 7.5 g
- Autoclave.
- When cool, add:

| | |
|---|---|
| PDM iron and vits | 10 mL |
| BAP | 0.5 mL |

- Pour into 15-100 mm petri dishes. (*This media can be stored for months.)*

**M2A (co-cultivation medium):** For 1L:

| | |
|---|---|
| PDM salts | 10 mL |
| $CaCl_2$ .$2H_2O$ | 0.2 g |
| Sucrose | 10 g |
| MES | 6 mL |

- Adjust the pH to 5.8 with KOH, then adjust the volume to 990 mL.
- (For solid media add Agar-agar 7.5 g)
- Autoclave.
- When cool, add:

| | |
|---|---|
| PDM iron and vits | 10 mL |
| BAP | 1.5 mL |
| NAA | 50 μL |
| Acetosyringone | 100 μL |

- Pour M2A-agar into 15-100 mm petri dishes. (*This media should be used within 4 weeks.)*

**M2R (regeneration medium):** For 1L:

| | |
|---|---|
| PDM salts | 10 mL |
| $CaCl_2$ .$2H_2O$ | 0.2 g |
| Sucrose | 20 g |
| MES | 6 mL |

- Adjust the pH to 5.8 with KOH, then adjust the volume to 990 mL.
- Add Agar-agar 7.5 g
- Autoclave.
- When cool, add:

| | |
|---|---|
| PDM iron and vits | 10 mL |
| BAP | 1.5 mL |
| NAA | 50 μL |
| Carbenicillin (to kill Agro) | 500 μL |

Selection agent: Km (25 mg/L) or Bialaphos (1 mg/L)

- Pour into 20-100 mm petri dishes. (*This media should be used within 4 weeks.)*

**M3 (Shoot development medium):** For 1L:

| | |
|---|---|
| PDM salts | 10 mL |
| $CaCl_2 .2H_2O$ | 0.2 g |
| Sucrose | 20 g |
| MES | 6 mL |

Adjust the pH to 5.8 with KOH, then adjust the volume to 990 mL.

Add Agar-agar 7.5 g

Autoclave.

When cool, add:

| | |
|---|---|
| PDM iron and vits | 10 mL |
| NAA | 50 µL |
| Carbenicillin | 500 µL |

Kanamycin (25 mg/L) or Bialaphos (1 mg/L)

Pour into magenta boxes. (*This media should be used within 4 weeks).*

**M4 (Rooting medium):** For 1L:

| | |
|---|---|
| PDM salts | 10 mL |
| $CaCl_2 .2H_2O$ | 0.2 g |
| Sucrose | 10 g |
| MES | 6 mL |

Adjust the pH to 6.5 with KOH, then adjust the volume to 990 mL.

Add Phytagel (gelgro) 3 g

Autoclave.

When cool, add:

| | |
|---|---|
| PDM iron and vits | 10 mL |
| IBA = 2.5 µM | 250 µL |
| Carbenicillin | 500 µL |

**No Kanamycin or Bialaphos**

Pour into magenta boxes (*This media should be used within 4 weeks).*

***Note: If no roots have formed after 2 weeks on fresh M4, re-cut the shoot and transfer to fresh M4.***