Video Article

Protocols for Obtaining Zygotic and Somatic Embryos for Studying the Regulation of Early Embryo Development in the Model Legume *Medicago truncatula*

Sergey Kurdyukov[1], Youhong Song[1], Terence W-Y Tiew[1], Xin-Ding Wang[1], Kim E. Nolan[1], Ray J. Rose[1]

[1]School of Environmental and Life Sciences, University of Newcastle, Callaghan, New South Wales, Australia

Correspondence to: Ray J. Rose at ray.rose@newcastle.edu.au

URL: http://www.jove.com/video/52635
DOI: doi:10.3791/52635

Keywords: Developmental Biology, Issue 100, Zygotic embryogenesis, somatic embryogenesis, developmental biology, tissue culture, *Medicago truncatula* , plant embryogenesis, legumes, embryogenesis, plant development, plant tissue culture

Date Published: 6/9/2015

Citation: Kurdyukov, S., Song, Y., Tiew, T.W.Y., Wang, X.D., Nolan, K.E., Rose, R.J. Protocols for Obtaining Zygotic and Somatic Embryos for Studying the Regulation of Early Embryo Development in the Model Legume *Medicago truncatula*. *J. Vis. Exp.* (100), e52635, doi:10.3791/52635 (2015).

Abstract

Early embryogenesis starting from a single cell zygote goes through rapid cell division and morphogenesis, and is morphologically characterized by pre-globular, globular, heart, torpedo and cotyledon stages. This progressive development is under the tight regulation of a complex molecular network. Harvesting sufficient early embryos at a similar stage of development is essential for investigating the cellular and molecular regulation of early embryogenesis. This is not straightforward since early embryogenesis undergoes rapid morphogenesis in a short while *e.g.* 8 days for *Medicago truncatula* to reach the early cotyledon stage. Here, we address the issue by two approaches. The first one establishes a linkage between embryo development and pod morphology in helping indicate the stage of the zygotic embryo. This is particularly based on the number of pod spirals and development of the spines. An alternative way to complement the *in vivo* studies is via culturing leaf explants to produce somatic embryos. The medium includes an unusual hormone combination - an auxin (1-naphthaleneacetic acid), a cytokinin (6-benzylaminopurine), abscisic acid and gibberellic acid. The different stages can be discerned growing out of the callus without dissection.

Video Link

The video component of this article can be found at http://www.jove.com/video/52635/

Introduction

Legumes are the third largest family of higher plants with approximately 20,000 species and the Leguminosae (or Fabaceae) family are second to cereals in area harvested and total production[1]. Soybean is the third largest cultivated crop. Grain legumes provide about one-third of dietary protein and one-third of vegetable oil for human consumption[2]. Legumes with their N_2 fixing capacity also contribute to sustainable agricultural systems. *Medicago truncatula*, like soybean, stores protein and oil in the cotyledons of its seeds and is a genetic and genomic legume model with considerable genetic and genomic resources[3,4]. While *M. truncatula* has enabled advances in understanding the legume-rhizobium symbiosis[4] it has been increasingly employed to study legume seed biology[5-7] and embryogenesis[8,9]. Arabidopsis embryogenesis has been extensively studied[10,11] but it is a non-legume and the details of embryogenesis are not identical to Medicago[8,10]. Zygotic embryogenesis in *M. truncatula* has interesting features, with a distinctive multicellular hypophysis, an endoployploid suspensor and basal transfer cell[8].

Somatic embryogenesis (SE) is commonly used for regenerating plants[12]. In the legume model *M. truncatula*, the seed line Jemalong 2HA (2HA) has been developed from the parent Jemalong to have high rates of somatic embryogenesis[13]. The number of embryos produced has recently been substantively increased by adding both gibberellic acid (GA) and abscisic acid (ABA) to the long established medium[14]. In this case GA and ABA act synergistically, which is unusual given that GA and ABA usually act antagonistically[14]. The embryos produced from callus develop on the surface which allows the stage of embryogenesis to be readily determined visually and readily harvested. Having near isogenic lines that are embryogenic (2HA) and non-embryogenic (Jemalong) facilitates the investigation of somatic embryogenesis and having both *in vivo* and *in vitro* systems provides different experimental possibilities.

Understanding the cellular and molecular mechanisms of embryo development is essential for understanding seed and plant development. In legumes, as in other dicotyledons, it is the cotyledons of the embryo that store the products that are used for human nutrition. Early embryogenesis involves rapid cell division, and correct embryo patterning. In approximately 8 days after fertilization, the *M. truncatula* embryo reaches early cotyledon stages. The morphological characterization is not exactly indicated by days after fertilization in glasshouse conditions. Thus, an efficient standardized approach to indicate the stage of developing embryos is valuable in studying the genetic regulation of early zygotic embryogenesis.

In this paper, we provide two standardized protocols to collect developing embryos for biological studies of embryogenesis in the legume model *M. truncatula*. The first one is to collect zygotic embryos by associating embryogenesis and pod morphology while the second is somatic embryogenesis via culturing leaf explants to provide easily accessed large embryo numbers.

Protocol

1. Zygotic Embryo Development

1. Plant Material
    1. Grow the *Medicago truncatula* wild type Jemalong or its near isogenic, highly re-generable genotype Jemalong 2HA[13] (known as 2HA) in a glasshouse with a 14 hr photoperiod and 23 °C/19 °C day/night temperature.
        1. Pierce the surface of the seed coat (with a 23 G needle) prior to sowing the seed so that water is allowed to enter the seed and soak in water overnight. Add enough water to fully cover the seed.
        2. Sow 3 seeds in each 15 cm diameter pot (total of 10 pots) in potting mixture (coarse sand, perlite, coir-peat (1:1:1) plus 5 g of Osmocote Exact Standard: slow release fertilizer) and transfer to the glasshouse. Retain the healthiest seedling after germination so there is 1 plant per pot and surround by a trellis for the plant stems to climb.

2. Harvesting Pods
    1. Collect pods from the wild type Jemalong or 2HA to obtain the appropriate early embryo stages as first described[9].
    NOTE: The different pod stages are shown in **Figure 1** and the corresponding embryo stages are shown in **Figure 2**.
    2. Check the different pod stages at harvest using criteria as per **Table 1**. Measure the time elapsed from stage 6 to help separate stages 6 and 7 (**Table 2**).

3. Dissecting ovules from pods and checking the embryo stage
    1. Isolate ovules from pods using fine forceps alone or a stereo dissecting microscope and forceps as necessary. If required the embryo stage can be quickly checked using a standard compound microscope (**Figure 3B**).
    2. Prepare modified Hoyer's solution containing 7.5 g gum Arabic, 100 g chloral hydrate, 5 ml glycerol, 60 ml deionized distilled water. Dissolve by stirring for 3-5 hr or overnight.
    3. For more refined microscopy clear the dissected ovules in modified Hoyer's solution[15,16]. Carefully pick up the intact ovules and place in a small volume of Hoyer's solution (enough to cover the ovule) at room temperature until clear.
    4. View the samples with differential interference contrast (DIC) optics. Capture the images iwth a digital camera[9] (**Figure 2**).
    5. Obtain the most uniform ovules from the central region of the pod and accumulate the required number. An example is shown in **Figure 3A**. Collect the ovules on ice and store at required temperature (-80 °C for nucleic acids) for further analysis.
    NOTE: For details on analysis of histological sections, transmission electron microscopy, and *in situ* hybridization please see papers[8,9].

2. Somatic Embryo Development *In Vitro*

1. Use an *M. truncatula* cultivar that is able to readily form embryos in culture. For this protocol for leaf explants use 2HA plants[13,17] that are 2-4 months old.
2. Take leaflets from the latest nearly fully expanded trifoliate leaf of an elongating stem.
3. Keep the trifoliate leaves on moist paper toweling in a culture pot to avoid dehydration.
NOTE: Once the trifoliate leaves are harvested they need to be utilized with minimal delay.
4. Preparation of the culture medium
    1. Use the P4 10:4:1:5 medium in agar (0.8% w:v) in 9 cm Petri dishes.
    NOTE: The P4 10:4:1:5 medium consists of the P4 basal medium[18] plus 10 µM 1-naphthaleneacetic acid (NAA), 4 µM 6-benzylaminopurine (BAP), 1 µM abscisic acid (ABA) and 5 µM gibberellic acid (GA). The use of GA + ABA accelerates embryo formation and causes substantive increases in embryo number[14].
    2. Make up the the P4 basal medium in 1 L; consisting of major salts (make calcium up separately), minor salts and vitamins (**Table 3**), chelated iron (make up separately), casamino acids (casein hydrolysate), 30 g sucrose, 8 g agar.
    3. Store stock solutions of major salts, calcium, casamino acids, minor salts and vitamins at -20 °C in suitable aliquots. Store chelated iron at 4 °C.
    4. Make up the chelated iron (200x) by dissolving 7.44 g of $Na_2EDTA•2H_2O$ in approximately 900 ml of deionized distilled water while stirring, then bring the solution to 98-99 °C while adding 1.853 g of $FeSO_4•7H_2O$. Finally make up to 1 L when the ingredients are dissolved. Store in amber colored bottles at 4 °C.
    5. Make up the P4 basal medium with the stock solutions as in **Table 4**. The hormones in the medium are 10 µM NAA, 4 µM BAP, 1 µM ABA, 5 µM GA (see **Table 4** and **Specific Materials Table**). Add the NAA and BAP prior to autoclaving and add ABA and GA after autoclaving and cooling to about 55 °C, using filter sterilization with a 0.22 µm filter attached to a syringe. Mix well by gentle swirling.
    6. Adjust the media to pH 5.8 with 1 M KOH prior to autoclaving. Autoclave at 121 °C for 20 min.
    7. After autoclaving add the filter sterilized ABA and GA, and pour approximately 25 ml of media into sterile 9 cm Petri dishes in a laminar flow hood or biohazard cabinet. Cool and let the agar set with the lid off. Then put on the lid and make sure there is no condensate on the lid. Label as required and store at 4 °C (in a cardboard box to prevent lid condensate).

5. Sterilization of Leaf Tissue
    1. Work in a UV-sterilized laminar flow hood or biohazard cabinet.
    2. Place leaves into the mesh ball of a previously autoclaved spring tea infuser.
    3. Immerse tea infuser containing the leaves in 70% (v:v) ethanol in a culture pot for 30 sec.

NOTE: For all steps in this procedure use 250 ml screw cap polycarbonate culture pots that are autoclaved.
4. Drain and then transfer and immerse the leaf tissue in 1:8 (v:v) diluted bleach (0.5% chlorine) for 10 min. Swirl gently from time to time.
5. Drain the bleach and transfer the tea infuser in a culture pot containing sterile deionized distilled water. Gently swirl and drain excess water. Repeat one more time with a fresh culture pot of sterile deionized distilled water.
6. Remove leaves from the tea infuser with sterile forceps to a fresh culture pot of sterile deionized distilled water. Screw on the sterile cap and rinse by inverting and swirling. Leave leaves floating on the sterile water until ready to prepare explants.

6. Preparing and Plating Explants
   1. Using sterile techniques, trim individual sterilized leaflets from the edge with a scalpel, and cut the remaining rectangle into two or three rectangular explants (8-10 x 3-5 mm) with the mid-vein in the center of each explant (**Figure 5A**).
      NOTE: The size of the explant is quite important in initiating the first callusing. Carry out the cutting on sterile lids from take-away food containers.
   2. Plate 6 explants on agar-solidified culture medium in 9 cm diameter Petri dishes (**Figure 5B**). Plate the explants abaxial side down to maintain usual leaf orientation.
   3. Seal the Petri dishes with Parafilm (**Figure 5C**) stretched around the dish. The tissue is now ready for incubation.

7. Tissue Incubation
   1. Incubate the plates in the dark at 28 °C in a controlled temperature room or growth cabinet throughout the culture period.
      NOTE: The explants will initiate dedifferentiation, undergo cell division, proliferation (callus formation) and embryogenesis (differentiation).
   2. Subculture the differentiating explants after 3 weeks on to fresh medium. At this subculture transfer the whole explant using a sterile stainless steel spatula and forceps, in a laminar flow hood or a biohazard cabinet. As callusing occurs and the callus exceeds the size of the largest one in **Figure 5C**, transfer 50% of the individual callus when subculturing to fresh medium.
   3. Observe the first embryos in about 4 weeks. While there is a degree of synchrony, collect different embryo stages over a 4-8 week incubation period (**Figure 6A,B**). Collect under a dissecting microscope and process according to experimental objectives.
   4. Subculture every 3 weeks and remove embryos periodically so that the explants will continue to produce embryos for 3 months.

Representative Results

For zygotic embryogenesis different pod structures corresponding to the different embryo stages are shown in **Figure 1A**-**F** while the different embryo stages are shown in **Figure 2A**-**F**. By selecting pods at the same stage, samples of ovules that are quite uniform can be obtained (**Figure 3A**). By using RT-qPCR embryo specific genes can be readily detected and time course studies evaluated[9]. Some additional dissection will allow for further enrichment of the embryo (**Figure 3B**). Processing for *in situ* hybridization strategies can be readily carried out as well as any complementary studies involving light (**Figure 4A, B**) and electron microscopy[8]. Of particular value in this system is the distinctiveness of the hypophysis and suspensor (**Figure 4A, B**).

For somatic embryogenesis the cutting of the initial explant from the individual folioles is shown in **Figure 5A**. The explants are then placed abaxial side up in close contact with the agar (**Figure 5B**). After callus formation embryos start to appear after 3-4 weeks and by 7 weeks there are numerous embryos at the cotyledon stage (**Figure 5C**). By following the plates through between weeks 4 and 7 embryos at discrete stages can be located (**Figure 6A, B**). The different embryo stages can be readily observed and simply picked off for analysis. This can be quite a quick way to gain certain types of information. For example **Figure 7** illustrates how embryos at the early cotyledon stage showed expression of one type of *MtOLEOSIN* gene but not another. Time courses studies of the gene of interest can then be examined using zygotic or somatic embryos. A particular advantage of the somatic embryo system is that transformation of the callus can be carried out without regenerating a whole plant and gene expression at different stages of embryogenesis visualized using GUS (β-glucuronidase) or fluorescent labels. An example is shown in **Figure 6C**.

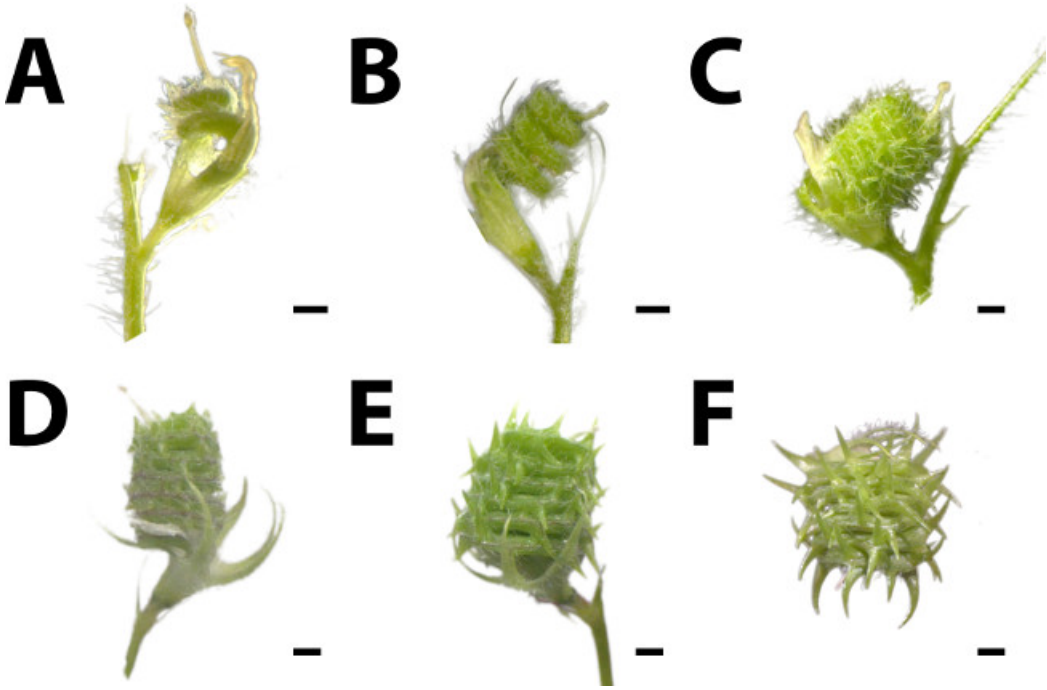

Figure 1: Pod morphology and embryo development stages. Pods at different stages of development with embryos at discrete stages. Stages 2-7 indicated. (**A**) and (**B**) are stages 2 and 3 very early and early pre-globular (**C**) stage 4 early globular (**D**) stage 5 late globular (**E**) stage 6 heart and (**F**) stage 7 late torpedo. Bar is 2 mm. Stage I is flowering (not shown).

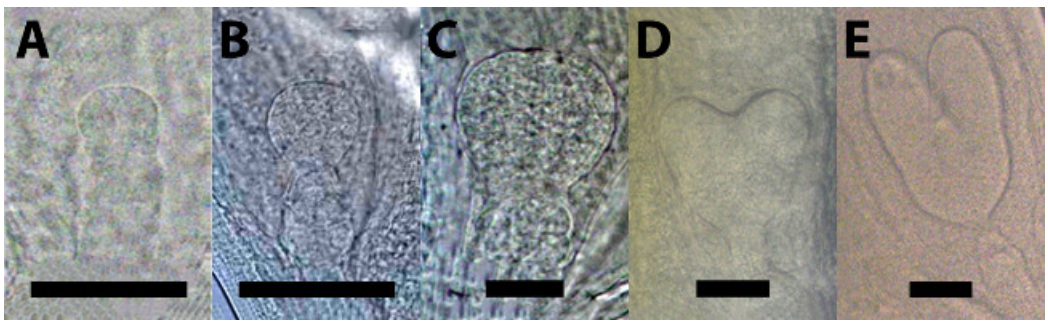

Figure 2: Embryo development stages. Cleared embryos at different development stages. (**A**) stage 3 early pre-globular, (**B**) stage 4 early globular, (**C**) stage 5 late globular, (**D**) stage 6 heart, and (**E**) stage 7 late torpedo. Bar is 60 µm.

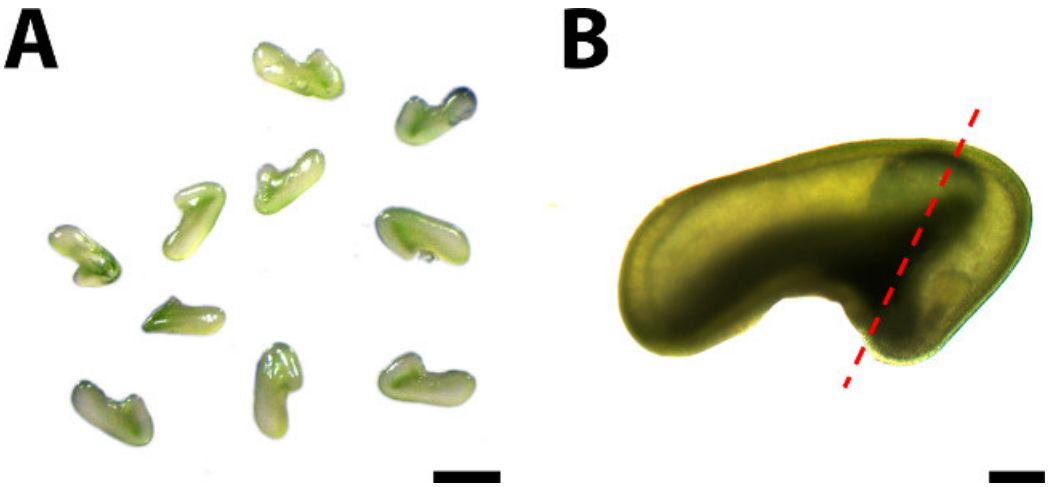

Figure 3: Isolated ovules. (**A**) Group of ovules with stage 5 embryos. (**B**) Ovule viewed under standard compound microscope to show embryo which can be excised at "hook" end. Bar is 1 mm (**A**) and 200 µm (**B**).

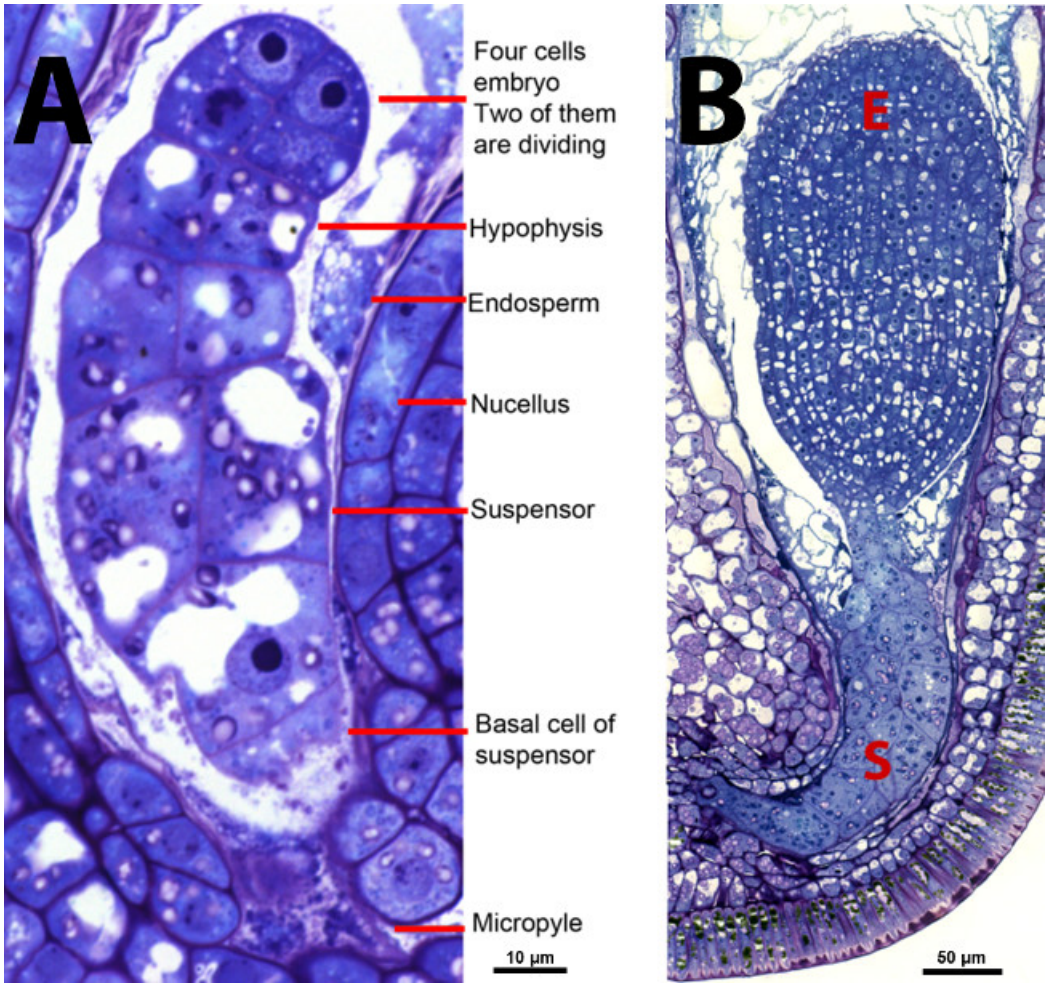

Figure 4: Morphology of the early embryo. (**A**) Section through very early embryo showing embryo proper (four cells), hypophysis (next four cells), suspensor (next four cells which have large vacuoles) and relationship to ovule tissues. (**B**) Section through early torpedo stage embryo (E) with prominent suspensor (S). Stained with toluidine blue. Bar is 10 µm (**A**) and 50 µm (**B**).

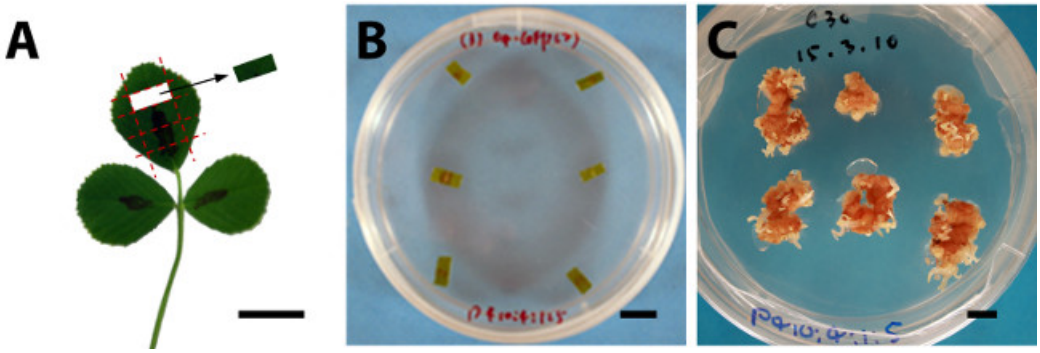

Figure 5: Culturing explants to produce somatic embryos. (**A**) Trifoliate leaf showing where explants from foliole are taken. (**B**) Explants plated on agar. (C) Somatic embryos produced from explants after 7 weeks culture. Bar is10 mm

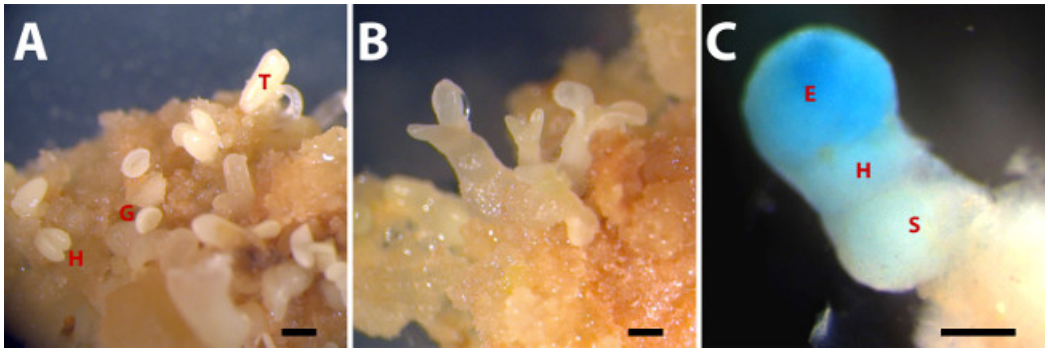

Figure 6: Somatic embryo stages and identifying the location of gene expression using transformation and GUS. (**A**) Somatic embryos at globular (G), heart (H) and torpedo (T) stages. (**B**) Somatic embryos at early cotyledon stage. (**C**) Globular stage somatic embryo showing strong GUS expression for the *MtWOX9* gene in embryo proper (E) and decreasing staining in hypophysis (H) and suspensor (S). Bar (**A**, **B**) is 100 µm. Bar (**C**) is 50 µm.

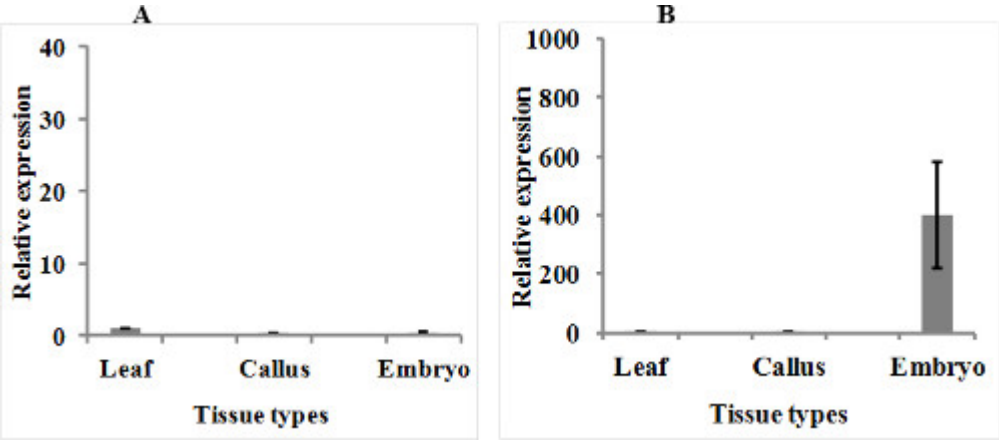

Figure 7: Gene expression duing embryogenesis *in vitro* using the quantitative polymerase chain reaction (qPCR). Gene expression of *OLEOSIN3* (**A**) and *OLEOSIN4* (**B**) in early cotyledon stage embryos excised from embryos from embryogenic callus. Expression is relative to leaf. Standard errors indicated.

Stage number	Embryo stage	Pod stage
Stage 1	Flower at anthesis	
Stage 2	Very early pre-globular embryo	Pod with one or two spirals
Stage 3	Early pre-globular embryo	Pod with three complete spirals
Stage 4	Early globular	Pod with five complete spirals and spines not visible
Stage 5	Late globular	Pod with six spirals and immature spines not exceeding pod width
Stage 6	Heart stage	Pod with six spirals and elongated maturing spines exceeding pod width
Stage 7	Torpedo stage	Pod with six spirals, mature thicker longer spines and increased girth

Table 1: Criteria for harvest of pod stages. Morphological criteria used in identifying pod stages corresponding to defined embryo stages.

	Flower Stage 1 (S1)	Pod Stage 2 (S2)	Pod Stage 3 (S3)	Pod Stage 4 (S4)	Pod Stage 5 (S5)	Pod Stage 6 (S6)	Pod Stage 7 (S7)
Days	0	1.5-2	0.5-1	0.5	0.5-1	1	1-1.5

Table 2. Time course for pod collection. The time from the previous embryo development stage indicated in days.

Major salts/L		Minor salts/L		Vitamins/L	
	mg		mg		mg
KNO_3	1875	$MnSO_4 \cdot H_2O$	10	Myo-Inositol	100
NH_4NO_3	600	H_3BO_3	3	Thiamine HCl	10
KH_2PO_4	131	$ZnSO_4 \cdot 7H_2O$	2	Nicotinic acid	1
KCL	225	KI	0.75	Pyridoxine HCl	1
$MgSO_4 \cdot 7H_2O$	225	$Na_2MoO_4 \cdot 2H_2O$	0.25		
Calcium separate		$CuSO_4 \cdot 5H_2O$	0.025		
$CaCl_2 \cdot 2H_2O$	300	$CoCl_2 \cdot 6H_2O$	0.025		
		Chelated iron separate			
		$FeSO_4 \cdot 7H_2O$	9.267		
		$Na_2EDTA \cdot 2H_2O$	37.2		

Table 3: P4 Medium. The components of the P4 medium.

Component	Amount/L
10 x major salts	100 ml
100 x calcium	10 ml
1,000 x minor salts	1 ml
200 x iron	5 ml
100 x casamino acids	10 ml
1,000 x vitamins	1 ml
Sucrose	30 g
Agar	8 g
Hormones	
1,000 µM NAA	10 ml
1,000 µM BAP	4 ml
1,000 µM ABA	1 ml
1,000 µM GA	5 ml

Table 4: Making up the P4 medium with hormones. Making up the P4 medium with hormones from stock solutions.

Discussion

The protocols described are relatively straight forward and allow investigation of legume embryogenesis with all the contemporary cell and molecular techniques. We recognize that there are advantages and disadvantages of both *in vivo* and *in vitro* approaches. Both allow more focus on early embryogenesis compared to culture of immature seeds[19].

In the case of *in vivo* studies what is described is predominantly the isolation of the ovule from the pod which is suitable for many embryo studies. It is of course possible to further enrich for embryo tissue by slicing off the “hook” area (**Figure 3B**). It is difficult to isolate the very earliest stage of embryo development as during disection the embryo is often lost as it is not well attached to the adjacent tissue, making the use of ovules advantageous. Using pod morphology eliminates the tagging of flowers and having a time course regime for each flower. In addition there is potential variability in developmental timing methods unless the environment is very tightly controlled. Optimum plant growth is important so that pod formation does not occur under stress conditions. Become familiar with pod development and slight adjustments can be made to define morphological stages to match specific embryo development stages.

In the case of somatic embryogenesis, it has to be recognized that the embryos are derived asexually and not from a zygote. Also, the nutritional source is different to the zygotic embryo. In case of the somatic embryo, it is the culture medium and surrounding callus, and in the case of the zygotic embryo it is the endosperm. This means the suspensor is much better developed in the case of the zygotic embryo (**Figure 4A, B**). The particular value of the *M. truncatula* system is the large number of embryos in response to the unusual four hormone regime. If the large numbers don’t occur then checking the details of making up the hormones and their addition to the medium should be checked. As with any culture methodology sterility of the explant tissue without damage (make small adjustments to timing of ethanol and hypochlorite sterilization times if this is a problem) is important together with the care in maintaining sterility during all preparative steps.

What we have found is that it is valuable to complement *in vivo* and *in vitro* processes. An example from our own studies is the investigation of the transcription factor MtSERF1 (SOMATIC EMBRYO RELATED FACTOR 1) which is an ethylene response transcription factor gene. MtSERF1

was first discovered in somatic embryos using RNAi, promoter-GUS fusions and in-situ hybridisations; and then shown to be expressed in zygotic embryogenesis[20].

The study of legume embryogenesis is important for human nutrition, and using *M. truncatula* embryos together with the cellular and molecular technologies now available can enhance this area. Insight can be obtained into how embryo size and hence yield can be optimized together with the required carbohydrate, oil and protein composition. These determinants are largely set in train in early embryogenesis[8,9].

Disclosures

The authors declare that they have no competing interests.

Acknowledgements

This research was supported by the Australian Research Council grant CEO348212 and the University of Newcastle. The assistance of Dr. Sam Zhang is acknowledged.

References

1. Gepts, P., et al., Legumes as a model plant family. Genomics for food and feed report of the cross-legume advances through genomics conference. *Plant Physiol*. **137** (4), 1228-1235, doi/10.1104/pp.105.060871 (2005).
2. Graham, P.H., and Vance, C.P. Legumes: importance and constraints to greater use. *Plant Physiol.* **131** (3), 872-877, doi/10.1104/pp.017004 (2003).
3. Young, N.D., and Udvardi, M. Translating *Medicago truncatula* genomics to crop legumes. *Curr. Opin. Plant Biol.* **12** (2), 193-201, doi 10.1016/j.pbi.1008.11.005 (2009).
4. Young, N.D. et al., The Medicago genome provides insights into the evolution of rhizobial symbioses. *Nature*. **480** (7378), 520-524, doi: 10.1038/nature10625 (2011).
5. Gallardo, K., Le Signor, C., Vandekerckhove, J., Thompson, R.D., Burstin, J. Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse metabolic processes related to reserve accumulation. *Plant Physiol.* **133** (2), 664-682, doi: 10.1104/pp.103.025254 (2003).
6. Verdier, J. et al., Gene expression profiling of *M. truncatula* transcription factors identifies putative regulators of grain legume seed filling. *Plant Mol. Biol.* **67** (6), 567-580, doi:1007/11103-008-9320-x (2008).
7. Thompson, R., Burstin, J., and Gallardo, K. Post-genomic studies of developmental processes in legume seeds. *Plant Physiol.* **151** (3), 1023-1029, doi: 10.1104/pp.109.143996 (2009).
8. Wang, X-D., Song, Y., Sheahan, M.B., Garg, M.L. and Rose, R.J. From embryo sac to oil and protein bodies: embryo development in the model legume *Medicago truncatula*. *New Phytol.* **193** (2), 327-338, doi:10.1111/j.1469-8137.2011.03925.x (2012).
9. Kurdyukov, S., Song, Y., Sheahan, M.B. and Rose, R.J. Transcriptional regulation of early embryo development in the model legume *Medicago truncatula*. *Plant Cell Rep.* **33** (2), 349-362, doi:10.1007/s00299-013-1535-x (2014).
10. Mansfield, S.G. and Briarty,L.G. Early embryogenesis in *Arabidopsis thaliana.* 2. The developing embryo. *Can. J. Botany.* **69**(3), 461-476, doi:10.1139/b91-063, (1991).
11. Seefried, W.F., Willman, M.R., Clausen, R.L. and Jenik, P.D. (2014) Global regulation of embryonic patterning in Arabidopsis by microRNAs. *Plant Physiol.* **165** (2), 670-687, doi:10.1104/pp.114.240846 (2014).
12. Birnbaum, K.D., Sánchez Alvarado, A. Slicing across kingdoms: regeneration in plants and animals. *Cell.* **132** (4), 697–710, doi:10.1016/j.cell.2008.01.040 (2008).
13. Rose, R.J., Nolan, K.E., and Bicego, L. The development of the highly regenerable seed line Jemalong 2HA for transformation of *Medicagotruncatula* - implications for regenerability via somatic embryogenesis. *J. Plant Physiol.* **155** (6), 788-791, doi:10.1016/S0176-1617(99)80097-2 (1999).
14. Nolan, K.E., Song, Y., Liao, S., Saeed, N., Zhang, X., and Rose, R.J. An unusual ABA and GA synergism increases somatic embryogenesis, facilitates its genetic analysis and improves transformation in *Medicago truncatula, PloS ONE.* **9**(6), e99908, doi:10.1371/journal.pone.0099908 (2014).
15. Liu, C.M. and Meinke, D.W. The titan mutants of Arabidopsis are disrupted in mitosis and cell cycle control during seed development. *Plant J.* **16** (1), 21-31, doi: 10.1046/j.1365-313x.1998.00236.x (1998).
16. Nolan,K.E., Kurdyukov, S. and Rose, R.J. Expression of the *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (*SERK1*) gene is associated with developmental change in the life cycle of the model legume *Medicago truncatula*. *J. Exp. Bot.* **60** (6), 1759-1771, doi:10.1093/jxb/erp046 (2009).
17. Iantcheva, A., Vlahova, M. and Atanassov, A. Somatic embryogenesis from leaf explants In: *The Medicago truncatula handbook.* Mathesius. **U,** *et al.* eds., Source: http://www.noble.org/MedicagoHandbook/ (2006).
18. Thomas, M.R., Johnson, L.B. and White, F.F. Selection of interspecific somatic hybrids of Medicago by using Agrobacterium transformed tissues. *Plant Sci.* **69** (2), 189-198, doi:10.1016/0168-9452(90)90117-7 (1990).
19. Ochatt, S.J. Immature seeds and embryos of *Medicago truncatula* cultured *in vitro*. In: *Plant Embryo Culture: Methodsand Protocols*, *Methods in Molecular Biology.* vol. 710, Thorpe,T.A. and Yeung, E.C. eds., Springer protocols, Humana press, 39-52 (2011).
20. Mantiri, F.R., Kurdyukov, S., Lohar, D.P., Sharopova, N., Saeed, N.A., Wang, X.D., VandenBosch, K.A. and Rose R.J. The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in *Medicago truncatula*. *Plant Physiol.* **146** (4), 1622-1636, doi10.1104/pp.107.110379 (2008).

