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Review:

Medicago truncatula as a model for understanding plant interactions with other organisms, plant development and stress biology: past, present and future

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Abstract. *Medicago truncatula* Gaertn. cv. Jemalong, a pasture species used in Australian agriculture, was first proposed as a model legume in 1990. Since that time *M. truncatula*, along with *Lotus japonicus* (Regal) Larsen, has contributed to major advances in understanding rhizobia Nod factor perception and the signalling pathway involved in nodule formation. Research using *M. truncatula* as a model has expanded beyond nodulation and the allied mycorrhizal research to investigate interactions with insect pests, plant pathogens and nematodes. In addition to biotic stresses the genetic mechanisms to ameliorate abiotic stresses such as salinity and drought are being investigated. Furthermore, *M. truncatula* is being used to increase understanding of plant development and cellular differentiation, with nodule differentiation providing a different perspective to organogenesis and meristem biology. This legume plant represents one of the major evolutionary success stories of plant adaptation to its environment, and it is particularly in understanding the capacity to integrate biotic and abiotic plant responses with plant growth and development that *M. truncatula* has an important role to play. The expanding genomic and genetic toolkit available with *M. truncatula* provides many opportunities for integrative biological research with a plant which is both a model for functional genomics and important in agricultural sustainability.

Additional keywords: abiotic stress, biotic stress, Jemalong 2HA, legumes, nodulation, regeneration.

Introduction

Medicago truncatula Gaertn. (barrel medic) is now well accepted as a model legume species, and, together with *Lotus japonicus* (Regal) Larsen, has helped to bring legumes into the position of being able to access many of the contemporary tools of genetics and functional genomics. Two recent demonstrations have emphasised the success obtained by a focus on model legumes. The activation (Gleason *et al*. 2006; Tirichine *et al*. 2006) of a calcium- and calmodulin-dependent protein kinase (CCaMK), and a gain of function mutation for the cytokinin receptor (Tirichine *et al*. 2007) Lotus histidine kinase 1 (LHK1), can cause the spontaneous formation of nodules independent of rhizobia. These latter results have also again raised the possibility of transfer of the rhizobia–legume symbiosis into other plant species (Oldroyd 2007). In this review, I wanted to consider the background to how *M. truncatula* became an important contributor to functional plant biology, particularly given its historical role in Australian agriculture, as well as its potential for integrating what were once considered discrete areas of investigation. *M. truncatula* is more than a model for plant–microbe interactions. In this context, I wanted to also highlight work on the interaction with pests and pathogens, plant development and stress biology. To maximise its reproductive success, the sessile flowering plant has to interact effectively with its biotic and abiotic environment as it grows and develops.

Medicago truncatula cv. Jemalong, agriculture and biotechnology

Annual *Medicago* species (annual medics) of Mediterranean origin have been particularly important in southern Australian agriculture because of their role in the wheat/sheep rotation. Cereal crops are alternated with legume pastures. The value of *M. truncatula* was recognised as early as 1939 and Jemalong was a commonly used cultivar (Crawford *et al*. 1989). This is an excellent example of sustainability with the pasture providing forage for livestock and symbiotically fixed nitrogen becoming available for subsequent crops (Loi *et al*. 2000). The annual medics germinate following the softening of hard seeds set in previous years (Crawford *et al*. 1989). More recently, a second generation of annual pasture legumes have been introduced to cater for a wider range of farming systems including grain legumes and oil seeds in phase farming systems (Loi *et al*. 2005).

With the advent of somatic hybridisation (Carlson *et al*. 1972) and then *Agrobacterium*-mediated transformation in 1983 (Bevan *et al*. 1983; Fraley *et al*. 1983; Herrera-Estrella *et al*. 1983; Murai *et al*. 1983), biotechnology was directed to many agricultural species including legumes. Regeneration was a requirement for these techniques. Regeneration from cultured tissues or protoplasts was conducted with the perennial, allogamous and autotetraploid *Medicago sativa* L., which proved amenable to regeneration via somatic embryogenesis from tissue explants (Saunders and Bingham 1972) and

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protoplasts (Johnson *et al*. 1981; Rose *et al*. 1986). Regeneration was restricted to special lines, notably Regen S, which was bred for regenerability (Bingham *et al*. 1975). Annual *Medicago* species were difficult to regenerate, but work by Bingham and co-workers indicated that the *Medicago* genus included species with 'regenerability' genes. *M. truncatula*, an annual autogamous diploid, is more attractive for transformation work and the associated genetics than the allogamous autotraploid *M. sativa*. The cultivar Jemalong proved amenable to regeneration but at extremely low frequency. What was surprising was that the few plants that regenerated, when used as explants, had a huge increase in regenerability (500×) via somatic embryogenesis and this was inherited (Nolan *et al*. 1989) and lead to the development of the highly regenerable Jemalong 2HA (2HA) (Rose *et al*. 1999). Using 2HA, transformation (Thomas *et al*. 1992; Chabaud *et al*. 1996; Wang *et al*. 1996), regeneration from protoplasts (Rose and Nolan 1995), asymmetric somatic hybridisation (Tian and Rose 1999) and transfer of agriculturally important genes such as viral resistance genes was feasible (Jayasena *et al*. 2001). Use of transformation as a tool for analysing gene function became crucial as whole-genome sequencing, large scale mutant isolation and the era of functional genomics began.

Medicago truncatula as a model legume

The foremost plant model *Arabidopsis thaliana* (L.) Heynh has many advantages for plant genomics, with its small size, short generation time, large numbers of offspring and small nuclear genome. Sequencing commenced in 1996; and its sequence was published in December 2000 (The Arabidopsis Genome Initiative 2000). The value of *Arabidopsis* mutants for functional analysis was shown some years ago by the isolation of mutants of C_3 species with defects in CO_2 assimilation and photorespiration (Somerville and Ogren 1979). The ability to transform *Arabidopsis* by the floral dip method (Clough and Bent 1998) has greatly facilitated research in *Arabidopsis* by enabling genome-wide insertional mutagenesis with T-DNA insertions (Alonso *et al*. 2003) to potentially enable knockouts of all genes. *Arabidopsis* is a dicotyledonous but non-nitrogen-fixing plant, making it important to develop a legume model with its ability to establish symbiotic interactions with rhizobia and mycorrhizae. With more than 18 000 species, legumes are the third largest family of higher plants (Young *et al*. 2003) and their ability to utilise atmospheric nitrogen fixation by the rhizobia symbiosis is of key importance in the biosphere and in agricultural systems.

In 1990, Barker *et al*. (1990) proposed *M. truncatula* as a model legume for the study of the molecular genetics of the rhizobia-legume symbiosis. *M. truncatula* ($2n = 2x = 16$) is diploid and autogamous, has a relatively small genome (~500 mbp – Bennett and Leitch 1995) and a generation time of ~3 months in long day conditions (Barker *et al*. 1990). These latter characteristics make it a more suitable model than the allogamous and perennial autotetraploid, lucerne, but like *M. sativa* it is efficiently nodulated by *Sinorhizobium meliloti* (Barker *et al*. 1990). The *Sinorhizobium meliloti* genome has now been sequenced (Galibert *et al*. 2001). A key reason for the choice of Jemalong in the Barker *et al*. (1990) proposal was its regenerability, which is relatively rare amongst annual legumes; and their preliminary work on transformation with *Agrobacterium.* My laboratory initiated work with *M. truncatula* cv. Jemalong in 1987 as it was the only *M. truncatula* cultivar that showed some regenerability. The regenerability was greatly enhanced by a cycle of tissue culture (Nolan *et al*. 1989) and subsequent selection for regenerability through the seed line lead to the development of Jemalong 2HA (2HA, Rose *et al*. 1999). We reported the first *M. truncatula* transformation with leaf explants using *Agrobacterium rhizogenes* and the more effective *Agrobacterium tumefaciens* procedure (Thomas *et al*. 1992). Subsequently we published a procedure using *Agrobacterium tumefaciens* (Wang *et al*. 1996). Several other transformation protocols have subsequently been published (see review Rose *et al*. 2003; Crane *et al*. 2006). Chabaud *et al*. (2003) have studied the kinetics of 2HA transformation, compared *Agrobacterium* strains and have used different reporter genes. Transformed 2HA with a single T-DNA insertion produce T_1 seed that segregate in a 3 : 1 ratio (Wang *et al*. 1996; Chabaud *et al*. 2003).

Though plant transformation via somatic embryogenesis is very useful for RNAi and overexpression studies for functional genomics, it is still a relatively long process of 4–5 months (Chabaud *et al*. 2003), and really high throughput transformation comparable to *Arabidopsis* remains a difficulty. The 2HA Jemalong line is, nevertheless, valuable for routine transformation from leaf explants (Chabaud *et al*. 2007), molecular breeding (Jayasena *et al*. 2001), understanding somatic embryogenesis mechanisms (Nolan *et al*. 2003) and transposon mutagenesis (Ratet *et al*. 2006). The A17 Jemalong line which is being sequenced (Kulikova *et al*. 2001; Young *et al*. 2005) does not regenerate via somatic embryogenesis but can be transformed by organogenesis procedures (Trieu and Harrison 1996; Zhou *et al*. 2004). The recent Zhou *et al*. (2004) procedure requires dissecting out cotyledonary nodes from emerging seedlings and rigorous selection procedures to avoid untransformed shoots (Zhou *et al*. 2004).

What has been a very valuable transformation adjunct in *M. truncatula* and the study of the rhizobia and the arbuscular mycorrhizal (AM) symbiosis has been the use of *Agrobacterium rhizogenes* to form transformed roots on composite plants (Boisson-Dernier *et al*. 2001). This protocol involves the inoculation of sectioned seedling radicles which form the hairy roots of the composite plants. Antibiotics such as kanamycin can be used to select for co-transformation of hairy roots with introduced constructs. The hairy roots successfully form nodules after inoculation with *Sinorhizobium meliloti* and can be colonised by AM fungi. The transgenic roots can be generated rapidly in 2–3 weeks which has been a great aid to *M. truncatula* symbiosis research.

Medicago truncatula was a focus for several meetings and workshops in the United States and Europe in the 1990s to establish it as a model and initiate the development of the necessary genetic and genomic tools (Cook *et al*. 1997; Cook 1999). Expressed sequence tags (ESTs) were rapidly developed, beginning with the *M. truncatula* root hair enriched cDNA library (Covitz *et al*. 1998) producing 899 ESTs. There are now 227 000 *M. truncatula* ESTs on the The Gene Index Project database (http://compbio.dfci.harvard.edu/tgi/, accessed 15 December 2007). The first steps towards sequencing were taken when

Nam *et al*. (1999) produced the first BAC clones from Jemalong A17. The first published genetic map of *M. truncatula* was produced by Thoquet *et al*. (2002) using two homozygous lines selected from Jemalong (Jemalong 6 or J6) and the Algerian natural population DZA315. Thoquet *et al*. (2002) noted that the three Jemalong lines A17, J5 and J6 could be considered to have an identical genotype (but different to the highly regenerable Jemalong genotype 2HA). BAC clones were mapped to *M. truncatula* A17 pachytene chromosomes by fluorescent *in situ* hybridisation (FISH), (Kulikova *et al*. 2001; Choi *et al*. 2004*a*; Kulikova *et al*. 2004). In the Choi *et al*. (2004*a*) study the mapping population was derived from A17 × A20. The A20 genotype is an *M. truncatula* ecotype with nodulation characteristics similar to A17 and a dominant leaf spot phenotype (Penmetsa and Cook 2000). From this work, it was inferred that gene-rich regions were located in the euchromatin rich chromosome arms, with the heterochromatin located in the centromere and pericentromeric regions This meant that if BAC clones could be identified as gene rich then most of the genespace could be obtained by BAC-by-BAC sequencing (Young *et al*. 2005). This latter strategy of anchored, clone-by-clone sequencing (as opposed to whole-genome shotgun sequencing) has been pursued. Genome sequencing began in 2002 (Young *et al.* 2005) and has continued until the present time (http://www.medicago.org/genome/, accessed 15 December 2007). In the *M. truncatula* genome assembly version 1.0 (Mt1.0) nearly 2000 BACs have been sequenced representing ~186.2 Mbp of non-redundant genome sequence, about two-thirds of the gene rich space. The completed sequencing of the gene rich space is expected by the end of 2008. This latter information can be accessed on the publicly available databases (http://www.medicago.org/, accessed 15 December 2007). Physical and genetic maps are available on this latter site. The reference mapping population is A17 × A20 (Ané *et al*. 2008). Genome conservation between *M. truncatula* and crop and other legumes, including *Lotus japonicus*, has been examined (Choi *et al*. 2004*b*; Cannon *et al*. 2006). There is substantive synteny between *M. truncatula* and *M. sativa* (Thoquet *et al*. 2002; Choi *et al*. 2004*a*) and *M. truncatula* and *Pisum sativum* L. (Choi *et al*. 2004*b*; Aubert *et al*. 2006). These mapping studies will provide valuable information for fundamental research and translational research with crop legumes.

Genetic and genomic tools available in *M. truncatula*

As outlined above there are now large numbers of ESTs, an increasing amount of gene rich genome sequence and physical and genetic maps available for *M. truncatula*. The EST information (227 000 ESTs) has been assembled into 18 612 TCs (tentative consensus sequences) and 18 238 singleton ESTs (http://compbio.dfci.harvard.edu/tgi/, accessed 15 December 2007). Current estimate of gene number from the sequencing program in *M. truncatula* is 42 358 (http://www.medicago.org/, accessed 15 December 2007). The *M. truncatula* chloroplast genome, which contains only one copy of the inverted repeat, is also an active area of study (Shaver *et al*. 2008).

For functional genomics it is necessary to develop forward and reverse genetics tools. The first mutations affecting nodulation phenotypes were obtained in *M. truncatula* using γ-rays (Sagan *et al*. 1995) and ethylmethane sulfonate (Benaben *et al*. 1995; Penmetsa and Cook 1997). Mutants have been crucial to the progress in understanding the mechanism of nodulation. Several developmental mutants, other than nodulation, have also been isolated by Penmetsa and Cook (2000). Reverse genetic strategies to infer gene function based on induced variation within a specific gene sequence are also being pursued in *M. truncatula.* These are RNAi, TILLING (Targeting Induced Local Lesions in Genomes), *Tnt1* insertional mutagenesis (Ratet *et al*. 2006), and a fast neutron deletion mutagenesis based system. Using *Tnt1* retrotransposon-tagged mutants a leaf development gene *SINGLE LEAFLET1* has been identified (Wang *et al*.2008). The current status of these reverse genetic strategies has recently been discussed by Ané *et al*. (2008). We have recently used dexamethasone inducible RNAi (Mantiri *et al*. 2008) to identify *SOMATIC EMBRYO-RELATED FACTOR1* (*MtSERF1*).

Transcriptomics have been facilitated by the development of microarrays following the completion of the large scale EST projects. A spotted 16 K microarray of 70-mer oligos (http://www.noble.org/medicago/NSF/nsf.activities.html, accessed 15 December 2007) available in 2003 has been used in gene expression studies, for example in AM development (Hohnjec *et al*. 2006). The Affymetrix *Medicago* GeneChip became available in 2005 and includes 32 167 *M. truncatula* ESTs and 18 733 gene predictions from *M. truncatula* genome sequences, 1896 cDNAs from *M. sativa* and 8305 gene predictions from *Sinorhizobium meliloti* (Dandgeard) de Lajudie *et al*. (Tesfaye *et al*. 2006). Udvardi and colleagues (2007) have also developed high-throughput quantitative reverse transcriptase (qRT–PCR) analysis of transcription factors, which is more sensitive than DNA array hybridisation methods. These authors also discuss methods to identify transcription factor target genes in a non-biased, high throughput manner.

In parallel with the development of the genetic, genomic and transcriptomic resources has been the development of an increasing amount of proteomic data for *M. truncatula* (e.g. Mathesius *et al*. 2001; Watson *et al*. 2003; Imin *et al*. 2004, 2005). This latter technology relies on protein separations and mass spectrometry. Proteins separated on two dimensional gels and trypsin digested proteins can be characterised by peptide mass fingerprints by MALDI-TOF-MS and then identified from available sequence information on publicly available databases for *M. truncatula* (Imin *et al*. 2004). Using LC/MS/MS protein spots can be fragmented into peptides and sequence information generated from an individual peptide (Imin *et al*. 2004). High throughput LC/MS/MS is an alternative to gel separation of proteins (Millar *et al*. 2005).

With all the approaches now available for functional genomics there is increasing reliance on bioinformatic resources and there are recent publications that have tabulated these resources (Cannon *et al*. 2005; Stacey *et al*. 2006).

The interaction of *M. truncatula* with other organisms

The *M. truncatula* model has been central to the progress in unravelling the legume-rhizobia symbiosis but is also proving valuable in other areas. Outlined below are several areas where

the *M. truncatula* system is proving useful in enhancing understanding of how plants interact with symbionts, pests and pathogens.

The rhizobia–legume symbiosis

The focus on the two legume models, *M. truncatula* and *L. japonicus*, has enabled substantive progress in the understanding of how Nod factors are perceived and the signalling pathway that ultimately leads to nodule formation. As the focus of this review is *M. truncatula*, the schematic in Fig. 1 draws attention to key *M. truncatula* mutants in the developing understanding of nodulation. Progress on the mechanism of nodulation has been reviewed in recent times (Riely *et al*. 2004; Oldroyd and Downie 2006; Stacey *et al*. 2006) and includes the *Lotus* mutants. Identifying the receptors and linking the signalling through to the Ca^{2+} spiking and the apparent phosphorylation of the GRAS-type NSP1 and

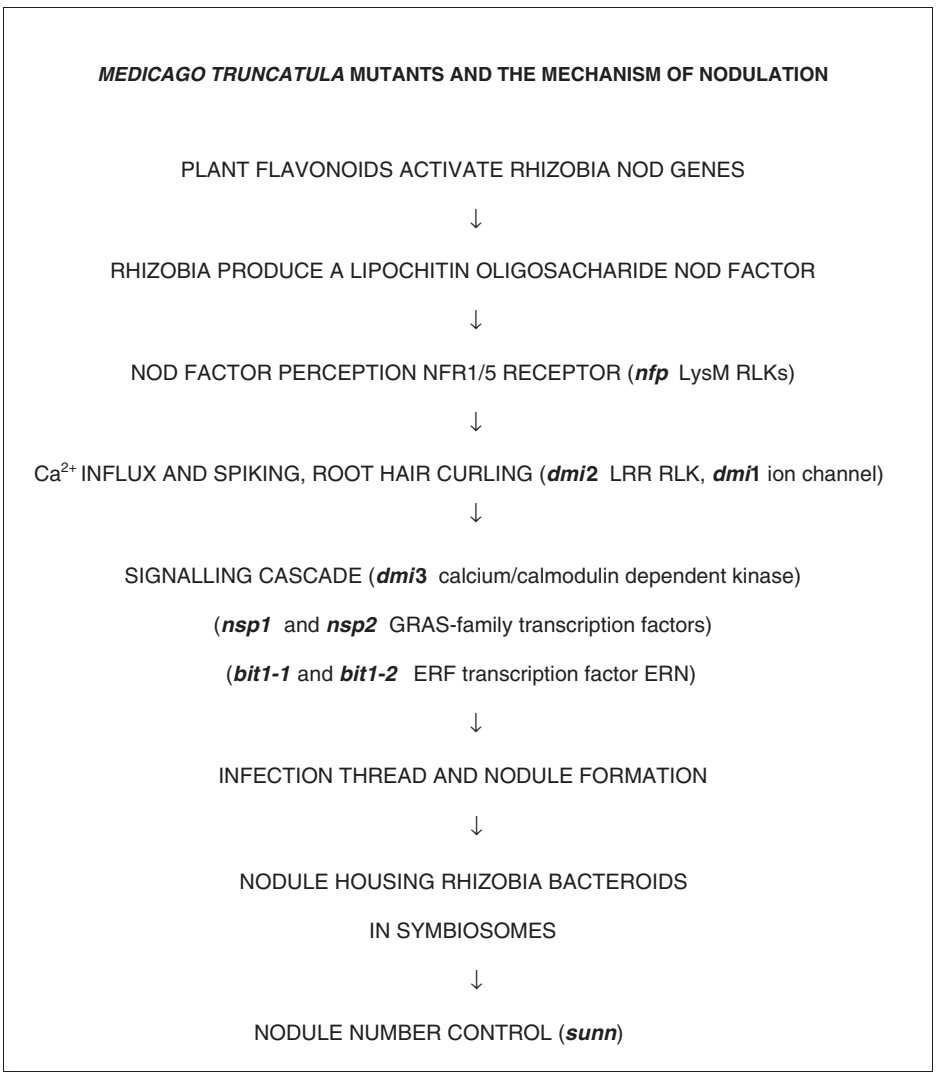

Fig. 1. Simplified diagram showing the contribution of key *Medicago truncatula* mutants to understanding the mechanism of nodulation. Information from Cullimore and Dénarié (2003) Riely *et al*. (2004), Udvardi and Scheible (2005), Oldroyd and Downie (2006), Stacey *et al*. (2006) and Oldroyd (2007). *nfp* is the *M. truncatula* NFP (NOD FACTOR PERCEPTION) locus (Amor *et al*. 2003), which mutates the LysM receptor-like kinases thought to be the Nod Factor Receptor NFR1/NFR5. *dmi2* (*doesn't make infection mutant 2*) mutates a plasma membrane located Leucine Rich Repeat Receptor Like Kinase (Limpens *et al*. 2005) which acts upstream of Ca^{2+} influx, Ca^{2+} spiking and root hair curling. *dmi1* mutates a ligand-gated cation channel (Ané *et al*. 2004). *dmi3* mutates a gene downstream of calcium spiking and is a calcium- and calmodulin- dependent protein kinase (Lévy *et al*. 2004). *nsp1* and *nsp2* mutate NODULATION SIGNALLING PATHWAY 1 (Smit *et al*. 2005) and 2 (Kaló *et al*. 2005), transcription factors of the GRAS family. *bit1–1* and *bit1–2* mutate the ERF transcription factor ERF REQUIRED FOR NODULATION (ERN) (Middleton *et al*. 2007). *sunn* mutates the *SUNN* gene which causes the hypernodulation phenotype (Schnabel *et al*. 2005).

2 transcription factors provides a strong framework for understanding nodule development. There are many steps in Fig. 1. to complete, particularly the integration of the development of the infection thread and the setting up of the symbiosomes associated with the morphogenesis of the nodule. Development of current thinking can be seen in several recent commentaries (Cullimore and Dénarié 2003; Udvardi and Scheible 2005; Oldroyd 2007).

The work on understanding signalling in nodulation is not only significant for understanding the mechanism of nodulation but also facilitates understanding of the AM symbiosis as well as interaction with pests such as nematodes. Further, the *M. truncatula* nodule is indeterminate producing an apical meristem. This provides a useful model to study organ morphogenesis and cell differentiation (Brewin 1991).

The arbuscular mycorrhizal legume symbiosis

Medicago truncatula has proven a useful model for investigating the arbuscular mycorrhizal symbiosis with the obligate biotroph *Glomus* spp. (Liu *et al*. 2003; Hohnjec *et al*. 2006). The AM symbiosis association enables a carbon supply for the fungus and an enhanced supply of mineral nutrients, notably phosphorus, to the plant (Liu *et al*. 2003; Hohnjec *et al*. 2006). The AM symbiosis as for the rhizobia symbiosis has important implications for ecology and sustainable agricultural systems. Using a legume model has the advantage of comparative studies of nodulation and mycorrhisation. Studies of mutants defective in nodulation have shown mechanistic similarities between nodulation and mycorrhizal infection (Stacey *et al*. 2006). Several *M. truncatula* mutants are defective in both the rhizobia and the AM symbiosis and the impaired genes are known as *SYM* genes (Parniske 2004). An example of these genes are the *DMI1*, *DMI2* and *DMI3* genes (Fig. 1), involved in both Nod factor and Myc factor signalling (Ané *et al*. 2004; Stacey *et al*. 2006). Though the rhizobia and AM response have some common signalling components, the NFR1 and NFR5 Nod-factor receptors are not the AM receptors (Parniske 2004). Transcriptional profiling studies using microarrays reveal that several hundred genes are upregulated during AM development and how these genes relate to current understanding of signalling remains to be explored (Hohnjec *et al*. 2006).

Harrison and co-workers (Harrison *et al*. 2002; Javot *et al*. 2007) have defined a *M. truncatula* phosphate transporter (MtPT4) that is essential for both the acquisition of phosphate delivered by the AM fungus and is a requirement for the AM symbiosis. The Javot *et al*. (2007) study used RNAi strategies to downregulate MtPT4, and MtPT4 loss-of-function mutants were identified by TILLING. It was shown that MtPT4 is a low affinity phospate transporter and is a member of a unique clade of phosphate transporters (Pht1, subfamily 1) which are only expressed in the AM symbiosis. The MtPT4 transporter is present in the *M. truncatula* periarbuscular membrane which forms a continuum with the plasma membrane of the *M. truncatula* cortical cell (Harrison *et al*. 2002).

The nematode–plant interaction

As noted above, there is overlap in the signalling pathways between rhizobia and AM symbionts and it is of interest to widen this comparison to include other microbe interactions such as with pathogenic nematodes (Mathesius 2003). The model legumes *M. truncatula* and *L. japonicus* can act as hosts for nematodes, which enables comparisons with the plant recognition of rhizobia and AM fungi and the subsequent signalling events (Bird 2004). Root knot nematodes (RKN) induce giant cells in the vascular cylinder where the RKN feed. There is evidence that nematode signalling at the root surface is influenced by mutations in Nod factor receptors (Bird 2004; Weerasinghe *et al*. 2005).

Plant–aphid interactions

Plant breeders have recognised for some time that annual *Medicago* species have a range of insect resistances and that there is a range of aphid resistance within *M. truncatula* cultivars and genotypes (Lake 1989). Development of *M. truncatula* as a model legume opened up genetic and molecular studies in this area (Klingler *et al*. 2006; Gao *et al*. 2008). Different resistance genes exist for spotted alfalfa (*Therioaphis trifolii* (Monell) f. *maculata*), pea (*Acyrthosiphon pisum* Harris) and blue green aphid (*Acyrthosiphon kondoi* Shinji) (Klingler *et al*. 2006; Gao *et al*. 2008). Using *M. truncatula*, Klingler *et al*. (2005) identified a single dominant gene which conferred resistance to the bluegreen aphid which is flanked by NBS-LRR resistance gene analogues. Salicylic acid- and ethylene-responsive genes were induced in both resistant and sensitive plants (Gao *et al*. 2007). However, 10 genes associated with jasmonate signalling were only induced in the aphid resistant line.

Medicago truncatula and pathogenic fungi

Medicago truncatula is also being increasingly used as a model to study pathogen resistance mechanisms (Ellwood *et al*. 2006*b*; Tivoli *et al*. 2006; Foster-Hartnett *et al*. 2007; Samac and Graham 2007). The value and basis of *M. truncatula* as a model for nectrotrophic pathogens has been reviewed by Tivoli *et al*. (2006). The group at the Australian Centre for Necrotrophic Fungal Pathogens is making use of the world's largest collection of *M. truncatula* accessions, curated by SARDI at the University of Adelaide Waite Campus. The collection has been shown to be highly diverse, with over 90% of individuals showing discrete genotypes (Ellwood *et al*. 2006*a*). Sources of resistance to *Phoma medicaginis* Malbr. & Roum. have been identified in the SARDI collection (Ellwood *et al*. 2006*b*). Legume powdery mildew caused by the biotroph *Erysiphe pisi* DC. has been investigated by Foster-Hartnett *et al*. (2007) using *M. truncatula* genotypes with different levels of resistance. This latter study includes microarray analysis of gene expression in three genotypes with moderate and high resistance, and susceptibility. This microarray analysis joins others on nodulation (Lohar *et al*. 2006) and AM infection (Hohnjec *et al*. 2006) and such data being accumulated will be valuable to link to each other and the mutant and single gene molecular studies. *M. truncatula* is also a useful pathosystem for root rot diseases caused by *Aphanomyces euteiches* Drechsler (Gaulin *et al*. 2007), for *Phytophthora* (Salzer *et al*. 2000) and for the soilborne bacterial wilt pathogen *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. (Vailleau *et al*. 2007).

Medicago truncatula and plant development

Nodulation

As well as being a structure that houses the rhizobia the legume nodule enables a suitable environment for nitrogenase to conduct symbiotic biological nitrogen fixation (Oldroyd 2007) and provides an interesting model for plant cell differentiation and morphogenesis. The nodule is initiated opposite xylem poles from differentiated cells of the inner cortex (Beveridge *et al*. 2007) which means that these cells have to dedifferentiate and re-enter the cell cycle. A primordium ultimately forms and a mature nodule is produced. In the case of *M. truncatula*, the indeterminate nodule forms an apical meristem from which the nodule develops. As with plant development in general the hormones auxin and cytokinin are key players in nodule morphogenesis (Beveridge *et al*. 2007). Evidence from *M. truncatula* RNA interference studies against the cytokinin receptor CRE1 has shown that cytokinin acts as a positive regulator of nodulation (Gonzalez-Rizzo *et al*. 2006). In *L. japonicus*, a gain of function mutation in the cytokinin receptor Lotus histidine kinase 1 triggers spontaneous nodule formation in the absence of rhizobia (Tirichine *et al*. 2007). An LHK1 loss of function mutant fails to initiate cortical cell divisions in response to rhizobial signalling, but infection thread formation occurs (Murray *et al*. 2007). It is argued that cytokinin is necessary and sufficient for dedifferentiation and cell proliferation leading to root nodule formation (Tirichine *et al*. 2007); so how is auxin involved in nodule development? Auxin is thought to be involved at different stages of nodule formation and is required for differentiation of the vasculature (de Billy *et al*. 2001). Auxin accumulates at the site of nodule initiation and it has been shown (Pii *et al*. 2007) that an increased rhizobia auxin synthesis promotes the formation of indeterminate nodules (as in *M. truncatula*) but not in determinate nodules.

Lateral root formation

Lateral root formation has been extensively studied in *Arabidopis* (Casimiro *et al*. 2003), but the use of legumes provides an interesting perspective as lateral root formation has some similarities to nodule morphogenesis (Beveridge *et al*. 2007). Lateral root primordia originate from pericycle cells and then the lateral root produces a meristem at the apex, as do indeterminate nodules (Beveridge *et al*. 2007). Auxin can stimulate lateral root formation in *Arabidopsis* (Hirota *et al*. 2007), and recent studies in *M. truncatula* with IAA-overproducing nodules showed increased lateral root formation (Pii *et al*. 2007). There may be an overlap between the early events in nodulation and lateral root formation (Beveridge *et al*. 2007); however, the response to cytokinin differs in nodulation and lateral root formation in *M. truncatula*. In the case of CRE1 RNAi knockdowns where nodulation did not occur there is strong additional evidence for cytokinin control of nodulation (Gonzalez-Rizzo *et al*. 2006), but in the case of lateral roots MtCRE1 RNAi roots showed enhanced lateral root density. Clearly, though there are conceptual similarities and some specific overlap between meristem ontogeny, the specific type of meristem produced ultimately requires the expression of a different gene set.

Somatic embryogenesis and organogenesis in vitro

Medicago truncatula forms an indeterminate nodule and conceptually it illustrates the ability of plant cells to dedifferentiate, re-enter the cell cycle and differentiate into an organ containing a meristem. *In vitro* systems in plant biology function in a conceptually similar way and offer the opportunity to investigate cellular differentiation mechanisms and to experimentally modify the system in different ways (e.g. through media additives). In the development of transformation techniques for *M. truncatula* lines were developed, such as 2HA, with a greatly enhanced somatic embryogenesis compared with wild type. By modifying the hormones it is possible to obtain adventitious roots in both 2HA and wild type. These developmental outcomes in response to hormones and genotype are shown in Fig. 2.

Somatic embryogenesis

Somatic embryogenesis, like nodulation, offers fundamental insights into differentiation mechanisms. Somatic embryogenesis is a natural occurring asexual reproductive process in some plants (Garcês *et al*. 2007). Regeneration by somatic embryogenesis is an important pathway for transformation in many species but as in *M. truncatula* is often restricted to a specific cultivar, although the reason for this is unknown. The understanding of somatic embryogenesis will also enhance our understanding of apomixis (asexual seed formation, where the genotype is the same as the mother plant) as well as zygotic embryogenesis. In this latter case, an important example is the *SERK1* gene, a leucine rich repeat receptor-like kinase, discovered in somatic embryogenesis (Schmidt *et al*. 1997) and expressed in apomictic (Tucker *et al*. 2003) and zygotic embryogenesis (Hecht *et al*. 2001). *MtSERK1* is important in somatic embryogenesis and also in *in vitro* auxin-induced root formation in *M. truncatula* (Nolan *et al*. 2003).

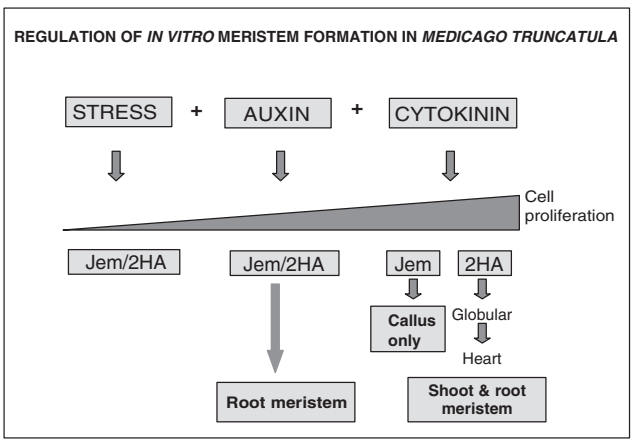

Fig. 2. Diagram of *in vitro* development of meristems in *Medicago truncatula* wild type Jemalong (Jem) and the Jemalong 2HA (2HA) embryogenic mutant. Cell proliferation of cultured leaf explants is initiated by the stress of excision and plating of the explant and increases in response to auxin, and is maximal in response to auxin plus cytokinin. Auxin stimulates root development in Jemalong and 2HA. The addition of cytokinin to the auxin medium inhibits root formation causing only proliferating callus in Jemalong and the bipolar embryos in 2HA callus. Based on Nolan and Rose (1998), Rose *et al*. (1999, 2006), Nolan *et al*. (2003) and Mantiri *et al*. (2008).

An unusual biological aspect of somatic embryogenesis in *M. truncatula* is that all the very highly regenerable genotypes have been derived after a cycle of tissue culture. These are Jemalong 2HA (Nolan *et al*. 1989; Rose *et al*. 1999), R108 (Hoffmann *et al*. 1997) and M9–10a (Araújo *et al*. 2004). This suggests that the regeneration process has consistently selected for a somatic cell with a somatic embryogenesis capacity, which is then inherited. As a cycle of tissue culture is always enough to enhance regenerability (Nolan *et al.* 1989), it suggests that the frequency is too high for a mutation and may be an epigenetic effect.

Somatic embryogenesis in *M. truncatula* requires a special genotype such as 2HA, auxin plus cytokinin plus the stress of the culture process, and research is in progress to define the signalling pathways involved (Rose and Nolan 2006). Isolated *M. truncatula* mesophyll protoplasts can regenerate via somatic embryogenesis and cell dedifferentiation and the first cell division cycle can be readily studied in these cells (Sheahan *et al*. 2005). In both *M. truncatula* and *Arabidopsis* massive mitochondrial fusion followed by fission is characteristic and relates to mitochondrial genetics (Sheahan *et al*. 2005).

Adventitious root formation in vitro

Adventitious root formation can be induced in cultured *M. truncatula* leaf explants by auxin in both 2HA and wild type (Nolan and Rose 1998; Fig. 2). Although this *in vitro* response has been known for 50 years in other species (Skoog and Miller 1957), it provides a useful system to study organ differentiation in the *M. truncatula* context. Histological examination of this system shows that root meristems are initiated *de novo* from procambial-like cells in the vasculature (Rose *et al*. 2006; Imin *et al*. 2007) and auxin-induced root formation is promoted in ethylene transduction mutants such as *sickle* which is most likely an *ein2* mutation. It is of interest to compare *in vitro* root formation regulation with that of nodules in *M. truncatula.* With the *sickle* mutant, there is stimulation of nodule number formation (Prayitno *et al*. 2006) which suggests that there is some commonality in committing pluripotent cells to a developmental pathway. In this later case ethylene is thought to modulate auxin transport (Prayitno *et al*. 2006). The value of organogenesis in an *in vitro* system is similar to that for embryogenesis in that it lends itself to media manipulation and the ready collection of material for high throughput analysis.

Seed development

Given the economic importance of grain legumes, seed development is an important area of investigation in this group of plants. A proteomic study of seed development in *M. truncatula* (cv. Jemalong line J5) at different stages of seed filling indicated the value of *M. truncatula* as a model for the analysis of seed filling in legumes (Gallardo *et al*. 2003). Studies by Djemel *et al*. (2005) on seed development and composition also concluded that *M. truncatula* was a suitable model for genomic approaches to seed development in grain legumes. During maturation protein and oil accumulated at fairly constant rates (Djemel *et al*. 2005), but the major protein groups were shown to accumulate in a specific temporal order (Gallardo *et al*. 2003). Firnhaber *et al*. (2005) have conducted microarray studies in flower pods and found more than 700 genes to be developmentally regulated.

Medicago truncatula and stress biology

Medicago truncatula is a valuable species to study how a plant interacts with its environment. It has the capacity to recognise signals from beneficial organisms and to have appropriate developmental responses. It also has to combat disease, pests and environmental stressors by recognising and responding to these signals to enable continuing growth and development. Soil salinity is a significant stressor for crop plants and adaptation of root development has been studied by Merchan *et al*. (2007) in *M. truncatula*. An AP2 transcription factor *MtZpt2–1* when overexpressed in *M. truncatula* plants carrying *Agrobacterium rhizogenes* transformed roots allowed sustained root growth under salt stress conditions. Another interesting feature of the study was that two genes homologous to cytokinin receptors were induced in the salt recovery phase. Hormones are known to be critical in root system growth and architecture and are important in responding to abiotic stress (Malamy 2005). In legumes it is interesting that cytokinin has a key role in nodule formation (Gonzalez-Rizzo *et al*. 2006) but inhibits root formation *in vitro* (Nolan and Rose 1998) and cytokinins in *M. truncatula* are negative regulators of lateral root formation (Gonzalez-Rizzo *et al*. 2006).

Drought tolerance investigations have also been carried out utilising *M. truncatula* genes. Zhang *et al*. (2005) have overexpressed *WXP1* a putative AP2 domain-containing transcription factor gene. This gene when overexpressed increased the cuticular wax and enhanced drought tolerance. It is noteworthy that MtZpt2–1 is also an AP2 transcription factor and has a single Ap2 domain which places it in the Dreb sub-family of the AP2/ERF super family. The AP2/ERF transcription family has some particularly interesting features as its members include genes related to abiotic and biotic stress and development (Nakano *et al*. 2006) which is suggestive of evolutionary forces connecting stress to development. Mantiri *et al*. (2008) have also recently found that a member of the ERF family of transcription factors is essential for somatic embryogenesis, possibly linking the stress of the culture process to development (Mantiri *et al*. 2008).

Transcription factors are clearly important regulators of both development and abiotic stress tolerance. In addition to the examples given, of *MtZpt2–1* and *WXP1* in relation to salt and drought stress, other legume transcription factors have been implicated in abiotic stress tolerance (Udvardi *et al*. 2007). As Udvardi and coauthors have pointed out (Udvardi *et al*. 2007), evolution has endowed plants with the ability to ensure their growth and development while fixed in space and subject to environmental extremes. Less than 1% of the more than 2000 transcription factors (TFs) in the model legumes (*Medicago* and *Lotus*) have been functionally characterised, so there is much scope to discover new strategies for using genetic means to influence stress tolerance (Udvardi *et al*. 2007). The Udvardi *et al*. (2007) legume transcription factor update has a lists of legume TFs that have been genetically characterised and those that have been characterised biochemically and

molecularly. Also there is a useful guide to domain shuffling between *Medicago* TF families in this latter article.

Conclusions and future prospects for research using *M. truncatula*

From the research discussed it can be seen that *M. truncatula* has emerged as an important model legume which has facilitated advances in the legume symbioses and opened up new areas of research into biotic stress, plant responses to pests and pathogens and plant development. There is a platform for continued progress in these areas. The completion of sequencing to capture most of the genes and the continued evolution of the bioinformatics is clearly important. As genome science progresses it seems likely that there will be a need to have a complete genome sequence, which given the advances in gene sequencing should be an attainable goal. *Arabidopsis* research has greatly benefited from the availability of insertional mutants which has made the identification of the function of all genes a realistic proposition. Though advances have been made, really high throughput transformation is still not possible in *M. truncatula.* There has been a promising recent study in *M. sativa* (Weeks *et al*. 2008) on *in planta* transformation directed at the apical meristem of the seedling, which are cut at the seedling node. Some specific areas where future *M. truncatula* research could provide further insights into legume and plant biology are highlighted below.

Can symbiosis be engineered?

With the increasing understanding of the signalling pathways involved in nodule and arbuscule signalling (Oldroyd and Downie 2006) and the demonstration that gain-of-function mutations in *CCaMK* and *LHK1* genes can cause spontaneous nodule formation in the absence of rhizobial bacteria (Gleason *et al*. 2006; Tirichine *et al*. 2007); the possibility of transferring symbiotic processes into other plant species has again been raised (Oldroyd 2007). Today, this possibility, with current genetic and genomic tools, increasingly looks a more realistic goal.

Small RNAs

There is another level of regulatory control that needs to be considered in understanding the regulation of plant processes. Gene expression can be regulated by RNA-induced gene silencing involving micro-RNAs (miRNAs) or short interfering RNAs (siRNAS) 21–24 nt in length (Jones-Rhoades *et al*. 2006; Axtell *et al*. 2007; Brosnan *et al*. 2007). The siRNAs and miRNAs guide argonaute-like proteins to mediate mRNA degredation, translational repression or transcriptional silencing (Jones-Rhoades *et al*. 2006; Brosnan *et al*. 2007). In plants, mRNA silencing can be transmitted from cell to cell and from roots to shoots (Brosnan *et al*. 2007). Many of the miRNAs regulate developmental processes (Jones-Rhoades *et al*. 2006). Small RNAs have received minimal attention in legumes.

Connecting plant growth and development to the abiotic and biotic environment

As highlighted in a recent opinion article (Potters *et al*. 2007), plants have to ultimately grow and develop out of trouble caused by environmental stressors. Legumes have this fascinating ability to have both symbiotic and defence responses leading to some different insights into the control of the diverse signalling pathways that contribute to the life of the legume plant. There are useful comparisons and overlaps in the area of plant–microbe interaction as well as in legume development (Beveridge *et al.* 2007). The biology of receptors is an area where functional overlaps are providing different perspectives. An example here is the SERK family of receptors (SERK3 is synonomous with BAK1) with roles in development, brassinosteroid reception and innate immunity (Chinchilla *et al*. 2007). One group of transcription factors that have interesting signalling connections when thinking of functional integration is the AP2/ERF superfamily of transcription factors (Alonso *et al*. 2003; Nakano *et al*. 2006). In *M. truncatula*, this family is involved in nodulation (Middleton *et al*. 2007), abiotic stress (Zhang *et al*. 2005) and development (Mantiri *et al*. 2008). In other species the role of the AP2/ERF superfamily in pathogen defence signalling is well established (Thatcher *et al*. 2005).

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