Investigation of gene expression underpinning partitioning of seed storage compounds in legumes

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Abstract
Legume seed store considerable protein and oil, and also starch in some species, however, the composition of storage compounds largely varies with the species. The molecular mechanism of seed storage partitioning remains to be clarified. In this paper, *Medicago truncatula* (the model legume) and *Medicago orbicularis* were used to compare the molecular basis underlying the partitioning of seed storage compounds. Seeds of *M. orbicularis* and *M. truncatula* were sampled at 10, 14, 18, 22 and 26 days after anthesis and at the time of maturation. *M. orbicularis* had a similar dry weight (4 mg per seed) to *M. truncatula*, but only accumulated around a third of seed storage protein and oil. The less seed protein and oil content was due to the lower expression of master transcription factors LEAFY COTYLEDON1-Like (L1L) and ABA INSENSITIVE 3 (ABI3), in *M. orbicularis*. *M. orbicualris* had similar expression of AGPase (a major enzyme controlling starch synthesis) to *M. truncatula*, suggesting that *M. orbicularis* did not switch carbon flow to the starch pathway. The findings in this study can be implied for molecular breeding or genetic transformation towards improving protein/oil production for food nutrition or biofuel purposes.

Key Words
Gene regulation, transcription factors, seed, protein and oil storage

Introduction
Legume seed accumulates considerable protein and oil, and also starch in some species such as pea and chickpea. The composition of seed storage compounds largely varies with legume species. The pathway to seed storage biosynthesis has been made clear, however, the mechanisms of controlling carbon flow into different storage compounds is still not well understood.

Many studies have shown that seed development and maturation are tightly regulated by transcriptional factors i.e. LEAFY COTYLEDON 1 (LEC1) or Like (L1L), LEAFY COTYLEDON 2 (LEC2), FUSCA3 (FUS3) and ABSCISIC ACID-INSENSITIVE3 (ABI3) (reviewed in Braybrook and Harada 2008). A common effect of the mutation of these genes is a striking reduction of seed protein and oil production (Meinke et al. 1994). These transcriptional factors function by binding (Yamamoto et al. 2009) on the promoter in storage proteins (Kroj et al. 2003) or enzymes of fatty acid biosynthesis (Mu et al. 2008). LEC1 or L1L, FUS3 and ABI3 have been identified in legumes based on ontological analysis (Verdier et al. 2008). *Medicago truncatula*, a legume model (Rose 2008; Thompson et al. 2009), contains about 10% oil in the mature seed (Wang et al. 2012). *Medicago orbicularis* has been reported to have 3% oil in the seed (Tonnet and Snudden 1974), which provides a natural species to be used in investigating the regulation of seed storage accumulation in legumes. *M. truncatula* has been well sequenced and well annotated (Thompson et al. 2009), and *M. orbicularis* is a very close species to *M. truncatula* (Tonnet and Snudden 1974), which facilitates to study gene expression comparisons in great details using a natural system. Therefore, this study compared the molecular basis during seed storage process between *M. truncatula* and *M. orbicularis*.

Methods
Plant growth and seed isolation
M. truncatula and *M. orbicularis* were grown in the glasshouse with a 14 h photoperiod, a 200-µmol/m/s^2 lighting intensity and 23/19°C day/night temperature regime. Flowers once fully open were tagged for use to indicate seed developmental stages. Seeds at 10, 14, 18, 22 and 26 days after anthesis (DAA) and when mature were isolated.

Protein and lipid assay
The determination of seed protein and oil content was described as Wang et al. (2012). Here we only introduced the methods briefly. Two seeds (5–10 mg) with three replicates at each stage were ground in 500 µL

of extraction buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 3 mg/mL polyvinylpyrrolidone and 10% (v/v) ethylene glycol (pH 7.5). After having extracted soluble protein, the insoluble protein was extracted by adding 500 µL of 1M NaOH into the pellet and vortexing for 15 min. Bradford assay method was used to measure soluble and insoluble protein. Lipid extraction was based on the method of Folch et al. (1957).

The seeds (50–100 mg) were homogenised with chloroform: methanol (2:1, v:v). After dispersion, the mixture was agitated for 15–20 min in an orbital shaker at room temperature. The homogenate was centrifuged to recover the liquid phase. The solvent was washed with 0.2 volumes of a 0.9% NaCl solution, vortexed and then centrifuged at 3000 g. The upper phase was discarded and the lower phase containing the lipids evaporated under a nitrogen stream. The weight of lipids was weighed and the oil content was calculated by dividing lipid weight with fresh weight of seeds.

Real time quantative PCR (qPCR)
RNA was extracted from growing seeds with three biological repeats using the RNAqueous-4PCR kit (Ambion). Genomic DNA was removed using DNase treatment after RNA isolated. cDNA synthesis was performed with a SuperScript III first-strand synthesis system (Invitrogen) using 1 µg of total RNA and oligo(dT) primers. Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3). The sequence of *M. truncatula* was from the database provided by phytozome 8.0 (http://phytozome.net/). The primers for *M. truncatula* were used to sequence selected genes with Australian Genome Research Facility (AGRF, http://www.agrf.org.au/). Once the existence of the gene had been confirmed in *M. orbicularis*, the comparisons of gene expression between species were then conducted. qPCR reactions were performed in triplicate (15-µL sample volume) using Platinum Taq PCR polymerase and 2-µM SYTO9 fluorescent dye (Invitrogen). Expression was calculated by relating to 10 DAA. Results shown are means ± standard error of three biological repeats.

Results and discussion
Protein and oil accumulation
The main storage proteins in legume seeds are globulin legumins and vicilins that both have a few isoforms. The accumulation of globulin storage protein and oil content and oil content of seed in *M. truncatula* and *M. orbicularis* are shown in Figure 1. The main storage protein in *M. orbicularis* was clearly lower than in *M. truncatula* (Figure 1a). *M. truncatula* seed contained 28% protein while *M. orbicualris* seed contained 8% protein only when the seed was fully mature.

Lipid accumulation in *M. orbiculars* was also far below that in *M. truncatula* during seed maturation. The seed accumulated 10% oil in *M. truncatula* and 3% oil in *M. orbicularis* when mature (Figure 1b), which was consistent with Djemel et al. (2005) and Tonnet and Snudden (1974). The variation of storage protein and oil in the two close Medicago species provided a natural system to investigate the gene regulation underlying the accumulation of storage compounds.

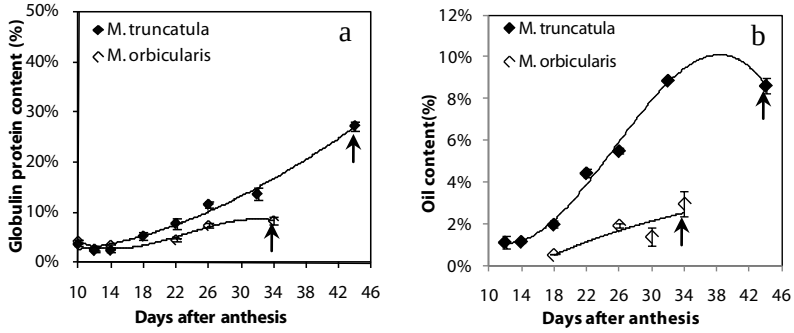

Figure 1. (a) Total protein and (b) oil accumulation during seed development in *M. truncatula and M. orbicularis* (Vertical bars indicate standard errors; arrow bars indicate mature stages).

Gene expression of master transcription factors
Time course of *LEC1*, *L1L*, *FUS3* and *ABI3* expression in *M. truncatula and M. orbiculari* is shown in Figure 2. Gene expression of *LEC1*, *L1L*, *FUS3* and *ABI3* followed similar patterns in both species during seed maturation. Little *LEC1* was expressed in either species (data not shown). *L1L* and *FUS3* peaked at 14 DAA and declined during seed maturation, while *ABI3* increased and reached a plateau at 18 DAA in *M. truncatula*,

and at 14 DAA in *M. orbiculari* (Figure 2). *L1L* was significantly lower from 10 and 18 DAA in *M. orbicularis* (Figure 2a), while ABI3 was significantly lower after 14 DAA (Figure 2c). *FUS3* expression was similar in both species (Figure 2b). The result suggested that *L1L* and *ABI3* were mainly responsible for the reduction of seed protein and oil in *M. orbicularis.*

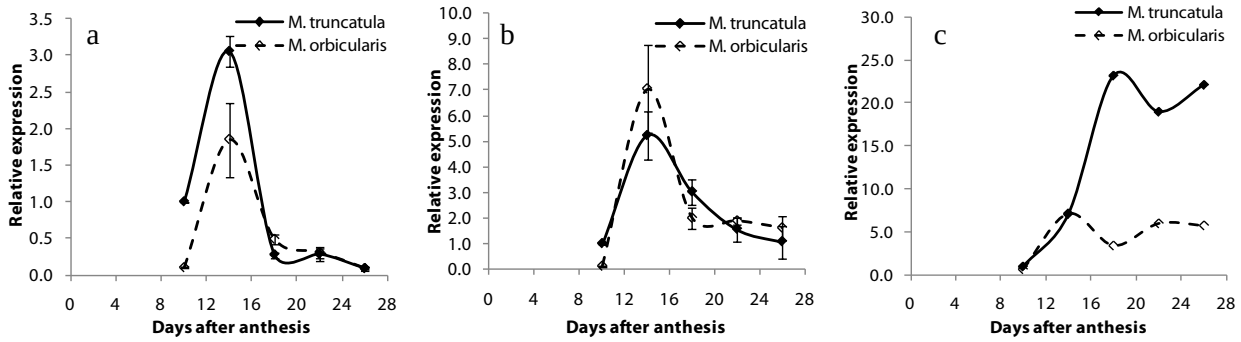

Figure 2. (a) *L1L*, (b) *FUS3* and (c) *ABI3* gene expression during seed development in *M. truncatula and M. orbicularis* (Vertical bars indicate standard errors).

Starch biosynthesis activity

The average seed dry weight was 4 mg/seed for both *Medicago* species, while protein and oil content in *M. truncatula* is much greater than in *M. orbicularis*. Here we checked if the lower protein and oil content would result in a switch on starch synthesis. ADP-glucose pyrophosphorylase (AGPase) has a central role in starch biosynthesis. It catalyses the first step of starch biosynthesis by generating the sugar nucleotide ADP-glucose from glucose 1-phosphate. Gene expression of a small subunit of AGPase enzyme i.e. Medtr3g107090 in *M. orbicularis* was higher before 16 DAA than *M. truncatula*, but the other small subunit i.e. Medtr5g104670 showed much lower expression (Figure 3), which suggested that AGPase activity in *M. orbicularis* was even weaker than in *M. truncatula*.

Further starch assay indicated that both species accumulated only a trace amount of starch with less than 2% of seed dry weight, but *M. orbicularis* was slightly lower (data not shown). This suggested that *M. orbicularis* might switch seed protein and oil to cell wall polysaccharides in cotyledon cells, which supports the finding that protein and oil was inversely related to cell wall polysaccharides (CWP) in soybean (Stombaugh et al. 2003). The measurement on content of cell wall polysaccharides for both *Medicago* species is necessary in the future.

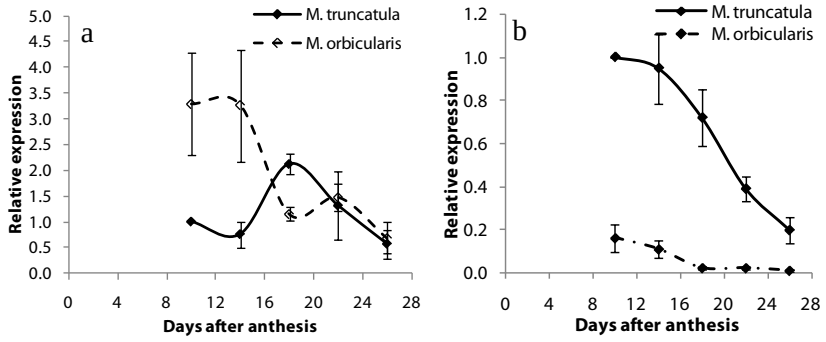

Figure 3. Gene expression of AGPase small subunits (a) Medtr3g107090 and (b) Medtr5g104670 during seed development in *M. truncatula and M. orbicularis* (Vertical bars indicate standard errors).

Conclusion

M. truncatula and another close species *M. orbicularis* showed different seed storage composition. Seed of *M. orbicularis* accumulated less protein and oil than that of *M. truncatula*. Gene expression data revealed that *L1L* and *ABI3* were most likely responsible for the lower content of storage protein and oil in *M. orbicularis*. *L1L* or *ABI3* can thus be the target for regulating the flow of storage compounds, for example, over-expression of *L1L* may result in more protein and oil accumulation. Compared to *M. truncatula*, *M. orbicularis* had a similar seed dry weight, but lower protein and oil content. In addition, both *Medicago* species had similar, low starch content. Together, the comparisons suggested that *M. orbicularis* might switch the synthesis of seed protein

and oil to other pathways such as biosynthesis of cell wall polysaccharides. The findings in this study can be used for directing molecular breeding or genetic transformation towards improving protein/oil production for food nutrition or biofuel use. Overall, this study has showed that *M. orbicularis* is a promising model to study the mechanisms of regulating carbon and nitrogen flow in legumes.

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