Seed Biology of *Medicago truncatula*

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Abstract

The seed of *Medicago truncatula* have morphological features typical of dicotyledons, and are borne in a spine-covered spiral pod. The cotyledons are rich in protein (35-45%), the major storage molecule, accumulate ca. 10% lipids and only traces of starch. The most abundant proteins are homologous to the globulin storage proteins of other legumes. The remainder of the dry weight is mainly composed of cell wall material and complex polysaccharides of the Raffinose family. The seed contains a residual endosperm constituting about 10% of the final seed mass. The embryo acquires desiccation tolerance by 12 DAP and afterwards is capable of germinating. Metabolite profiling in the endosperm, seed coat and embryo along with global approaches, such as transcriptomics and proteomics, were used to allow the dissection of molecular processes underlying seed development. An in vitro culture system has been developed allowing comparison of cotyledon development with or without the surrounding maternal seed tissues. Mature seeds exhibit dormancy that can be overcome e.g., by scarifying the seed. Seed size, seed dormancy and pod morphology are characters varying greatly between *Medicago* species, and characterization of genetic variability for these traits within *M. truncatula* is underway in several laboratories.

1 Introduction

The objective of this review is two-fold; to summarize the data currently available on the seed biology of *Medicago truncatula*, and to highlight those features that distinguish *M. truncatula* seeds from those of other legumes, and from higher plant seeds in general, which render the system particularly interesting.

*M. truncatula* seeds develop inside a pod that provides protection during development and is a source of nutrients, as well as possibly contributing to maintenance of dormancy (Baskin and Baskin, 1998). The *Medicago* genus exhibits a remarkable variation in pod structure based on variable extents of spiralling of the carpel during development and variations in disposition and extent of spine formation. The spines on the resulting mature pod favour mechanical dispersal, and the compressed spiral structures presumably contribute to protection of the mature seeds against herbivores.

*M. truncatula* displays a typical indeterminate growth habit, resulting in flower and seed set over a protracted period after the development of the primary inflorescence meristem. The plant is autogamous and sets seed efficiently in the absence of insect pollination. The only effective method for maximizing seed yield is to grow the plants under optimal conditions of nutrition and illumination. Despite a low harvest index, competition for assimilate between vegetative parts and the seed is shown by male-sterile lines, which grow indefinitely and accumulate considerable quantities of foliar biomass. Seed development following fertilization under controlled conditions proceeds at varying rates depending on the position of the seed in the pod, the most basal seeds developing more rapidly than the distal ones. To minimize the effects of heterochrony on time course analysis, it is advisable to harvest grains from one part of the pod only.

*Medicago* species have harvest indexes (HI) of ~10% in contrast to crop legumes which have HIs of 50% or more. Seed yield is important for seed producers, and studies on the genetic and ecophysiological control of seed production have been carried out in *M. sativa*. These indicated a large environmental and within-population variability compared to inter-population variability,
ie., the genetic base may be difficult to define within this material. Characters of importance appeared to be the number of seeds per pod and per inflorescence, with the suggestion of a role for the long peduncle locus. High seed yield was associated with high overall biomass production, i.e., not due to alterations in HI. There is considerable variation in seed size and number of seeds per pod (1-22) within the Medicago genus (Lesins and Lesins, 1979).

Genetic variability for seed protein content and composition was recently studied in 50 lines of *M. truncatula* derived from ecotypes or cultivars of diverse geographical origin grown under uniform conditions (Le Signor *et al.*, 2005). Seed protein content was highly variable among the 50 lines examined and the polymorphism for the major seed protein classes [the vicilin/convicilin (7S) and the legumin (11S) type proteins] allowed the clustering of the genotypes into four groups. All lines not belonging to either *M. truncatula* ssp. *truncatula* or ssp. *longispina* were clustered in a single group, demonstrating the value of seed protein profiles in delimiting species boundaries. The Jemalong line group was differentiated early in the dendrogram, and thus represents an ancient clade in the seed diversity of *M. truncatula*. As expected for an autogamous species, within-accession variation for protein composition was low. Lines contrasting for qualitative traits and seed protein content were identified to allow for the genetic determination of these characters, which will be relevant for legume crop improvement.

2 **Morphogenesis**

The cytology of seed development has been recently reported in two studies concentrating on features of the seed coat and the pod wall and on embryo and endosperm histodifferentiation (Wang and Grusak, 2005, Abirached-Darmency *et al* 2005). Four developmental stages between 3 and 20 days after pollination (DAP) were considered. At 3 DAP, the seed coat is already differentiated into 4 cell layers, the epidermis, hypodermis, parenchyma and nucellus running from outside inwards. The nucellar tissue is concentrated at the hilar end of the embryo sac.

In 6 DAP seeds the globular stage embryo, more easily distinguishable, is attached to the wall of the embryo sac by a well developed multicellular suspensor arranged in two or three cell rows in contrast to that of Arabidopsis (Fig 1a). The suspensor disappears by the torpedo stage. In 8-10 DAP seeds, the early torpedo embryo still occupies a relatively small fraction of the seed volume, the remainder of the embryo sac cavity being filled with endosperm which is already cellularized. The endosperm nuclei displayed the higher endoploidy level in the seed as shown by the size of DAPI-stained nuclei. The active proliferation of endosperm occurs before 12 DAP at the first phase of seed development. It remains prominent during the early maturation phase between 12 and 18 DAP occupying more than half of the seed volume (Fig 1b).
Figure 1

a) Nomarski image of a 6 DAP globular embryo with a prominent suspensor; b) Thick section of 14 DAP seed (mid embryogenesis) with extensive endosperm development; c) Thin section of a 12-14 DAP seed showing the structure of the seed coat at the micropyle region. Abbreviations: suspensor (su), embryo (emb), endoplasm (endo), micropyle (mi), palisade epidermis (pl), hypodermis (hy), vascular apparatus (va) ground parenchyma (gp), starch granule (sg).
Endosperm tissue was also detected in mature *M. truncatula* seeds unlike those of faba bean and pea where the coenocytic endosperm disappears before maturation (Borisjuk et al., 1995; 2002a). In *M. truncatula*, like in Arabidopsis, the endosperm may be a key player in seed development for the control of seed size as suggested by the analysis of *HAiku* and *TITAN* Arabidopsis mutants (Tzafir et al 2002, Garcia et al 2003). The ultrastructure of endosperm cells also revealed numerous plasmodesmata in the cell walls. With high concentration of mitochondria indicating active solute synthesis, these modifications support embryogenesis by furnishing metabolites, and perhaps by providing developmental signals, as shown in Arabidopsis and *M. sativa* (Berger et al 1999, Aivalakis et al 2004, Gallardo et al, 2006).

By 12–14 DAP the rate of cell division in the torpedo stage embryo has diminished and cellular expansion and storage accumulation begins. The cotyledons display a ramified provascular network, and an emergent embryonic axis. At this stage, a developmental gradient within the growing cotyledon is observed, reflected by differences in cell size. The cotyledon parenchyma showed a typical wave-like differentiation pattern similar to that reported for other legumes such as *V. faba* and *P. sativum*, in which differentiation begins in the adaxial region and spreads to the abaxial surface. DAPI staining shows that the outermost abaxial cells still maintain mitotic activity up to 16-18 DAP.

From 6 to 12 DAP water content of the seed remains high (~90%), and the seeds are unable to germinate or to withstand desiccation during this phase. The transition between the histodifferentiation phase and the seed filling phase, at around 14 dpp., marks a key switch in several aspects of seed development. The seeds begin to acquire germination ability, and the frequency of cell divisions diminishes. Most importantly, storage product accumulation begins. Seed coat sections at this stage reveal a multilayered structure (fig. 1c). No further cell division is observed, cell expansion alone causing further seed coat growth. Phloem is supplied to the seed via a single chalazal vascular bundle in the seed coat adjacent to the funiculus/hilum. It is well-developed by 13 DAP, and is organised in characteristic tracheids as in most Fabaceae seeds (Esau 1977, Van Dongen et al., 2003).

Under the mucilaginous layer, the palisade epidermis layer of the outer integument has developed macrosclereids containing lignified thickenings oriented perpendicular to the seed surface. Seed shape is determined by macrosclereid formation, lignification continuing up to 20 DAP. The hypodermis layer displays irregularly shaped cells with an extensive intercellular space, the osteosclereids, which increase significantly their wall thickness by 20 DAP. Adjacent is the ground parenchyma which contains abundant large amyloplasts with starch granules. The endothelium is the innermost seed coat layer consisting of smaller cells, with dense cytoplasm that accumulate anthocyanins. By 16 DAP the mucilaginous sheath develops the columella, due to the production of elaborate branched secondary cell walls associated with mucilage secretion. The columella has also been reported in Arabidopsis seed coat but its function is still unknown. The aleurone cell layer abuts the inner integument of the seed coat. A layer of epidermal-type cells has been identified, which enclose the embryo cavity, possibly with a transfer cell function. By the end of seed development at 30 DAP, when the surface of the mature embryo is in contact with the seed coat, certain cells are resorbed after compaction and the structure of the seed coat becomes progressively thinner. Although thin compared to that of many legume seeds, the seed coat of *M. truncatula* apparently confers a high measure of impermeability to the mature seed that contributes to dormancy.
During the course of seed filling (14-20 DAP), the cotyledon cells contain several protein storage vacuoles which have replaced the large central vacuole and can be revealed by naphtha photochemical staining. These dense bodies (1µm to 5µm diameter) are Fast Green positive and also detected with anti-vicilin antibodies. Some transient starch granules are also detectable inside amyloplasts of the cotyledon cells.

At mid-embryogenesis, in situ hybridization patterns of mRNA accumulation indicated that vicilin and legumin A gene expression was just beginning in the 14-16 DAP seed sections. The lower expression level of legumin at this stage corroborated proteomic evidence that legumin was accumulated later than vicilin (Gallardo et al., 2003). In situ hybridization indicated that transcription of vicilin and legumin A occurred mainly in the embryo storage parenchyma. Hybridization signals were never detected in the embryo epidermis or provascular region, the endosperm, or the seed coat. In 16 DAP seed sections, weak legumin mRNA signals were also observed in the embryonic axis cells, as reported in Arabidopsis for the 2S1 storage protein gene (Devic et al., 1996). After 20 DAP, the seed enters a maturation phase that involves the acquisition of desiccation tolerance, and water loss. Late embryogenesis abundant (LEA) proteins start to accumulate in the embryo at this stage, as generally observed in dicotyledonous seeds.

### 3 Storage compound accumulation

Early seed development in *M. truncatula* (morphogenesis phase) is characterized mainly by cell divisions. At around 12-13 days after flowering (DAP), cell divisions cease or slow down in the embryo, and endoreduplication takes place, heralding the seed filling phase. During seed filling and subsequent maturation, most of the dry weight (DW) increase occurs (Figure 2), the storage products accumulating mainly in embryo cells. *M. truncatula* seeds accumulate large quantities of protein at maturity (32-42% of dry weight; Djemel et al., 2005). The balance is 8-10% fatty acids and 6-10% of soluble sugars, the remainder being mainly complex polysaccharides. Starch content is less than 1%. Thus, seed composition is more similar to oilseed legumes such as soybean or lupin than it is to pea or field bean, despite being phylogenetically closer to these latter crop species. Many features of *M. truncatula* are characteristic of a plant that has not undergone selection during domestication, including the small seed size, difficulty of extraction from the pods, and the relatively high proportion of oligosaccharides belonging to the raffinose family, which are indigestible to humans.
Figure 2. Evolution of the embryo composition and embryo dry weight (DW) during *M. truncatula* seed development. Data were obtained for the ecotype Jemalong (inbred line J5). Embryo composition (%) is shown on the left axis, embryo DW as well as protein quantity per embryo are indicated on the right axis.

### 3.1 Storage protein synthesis

The storage protein complement of *M. truncatula* is typical for legumes of the Galegoid tribe (e.g. *Pisum sativum*, *Vicia faba*), consisting of representatives of the three major families, the legumin, vicilin and convicilin types, having similar sizes, amino acid compositions, and protein sequence homologies of 70-85% to those found in pea (Gallardo *et al.*, 2003). In addition, polypeptides related to the PA2 albumin type described for pea are present. The major storage protein classes accumulate in a sequential fashion during seed development, with the vicilin-class being followed by the legumin class and the convicilin class appearing last. The delay between the synthesis of each globulin class is approximately two days (Gallardo *et al.*, 2003). During an analysis of the kinetics of protein accumulation during seed development, storage protein precursors were also identified. These were detected in low quantity during seed filling with very similar time courses as the mature forms, indicating that synthesis and maturation of storage proteins are not developmentally separated in *M. truncatula*. The staging in synthesis of the different globulins appears to be transcriptionally controlled, as the corresponding mRNAs are also sequentially expressed. In addition to the storage proteins, a number of co-expressed proteins were identified that may be involved in the process of deposition, including a protein disulphide isomerase, a BIP-type chaperonin, and two 100 kDa precursor accumulating vesicle (PV-100) components (Gallardo *et al.*, 2003). These PV-100 proteins have been shown to be transported by vesicles to storage protein vacuoles, and to be processed there to liberate mature...
vicilin plus a basic cytotoxic-related peptide (Yamada et al., 1999) that may have an anti-herbivore role.

The contribution to storage protein accumulation in the embryo of the surrounding seed tissues has been demonstrated in vitro (Gallardo et al., 2006). When developed in the presence of an exogenous nitrogen supply, entire seeds and isolated embryos accumulate seed storage proteins. In the absence of added nitrogen, seeds and isolated embryos responded differently; with intact seeds there was a remobilisation of endogenous nitrogen during the initial stages of embryo development from tissues surrounding the embryo, thereby ensuring initial storage protein accumulation, whereas isolated embryos were unable to synthesize de novo storage proteins (Figure 3C-E). This phase of dependence appears to involve initial remobilisation from the integument, and subsequent remobilisation from the endosperm.

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**Figure 3.** Variation in globulin (vicilin and legumin chains) accumulation during *M. truncatula* seed development in planta and in vitro. A and B, in planta accumulation of globulins between 12 and 16 days after pollination (DAP), respectively. C, enlarged window from A or B showing the accumulation of globulins after development of the 12 DAP seeds for six days in vitro without nitrogen. D and E, enlarged window from A or B showing the contrasted profiles of isolated embryos collected at the 12 DAP stage and cultured in vitro in the absence (D) or presence (E) of nitrogen in the medium. In red, proteins identified by mass spectrometry. In blue, proteins not yet identified.
Evolution of storage composition in an embryo cell is presented in figure 2, along with increase in DW of the embryo. It is evident that protein metabolism and protein storage dominate primary metabolism in *M. truncatula* storage cells. Other significant storage compounds are lipids, oligosaccharides, and minerals.

### 3.2 Fatty acids

Fatty acid (FA) synthesis occurs from the very beginning of maturation. The concentration of FA (as methyl ester derivatives) which is ca 9% of DW at 13 DAP (days after flowering) increases to a maximum of 12% at 19 DAP where it stays until completion of dry matter accumulation (Djemel *et al.*, 2005). Palmitate (C16:0), linoleate (C18:2) and linolenate (C18:3) were the main FA found in the very young embryo. The beginning of maturation around 13 DAP is marked by a sharp decrease in the pool of saturated FA and a concomitant increase of the proportion of polyunsaturated FA, especially C18:3. In the mature seed about 75% of FA are incorporated into triacylglycerides and four of them, namely C16:0 (22%), C18:1 (16%), C18:2 (29%) and C18:3 (27%), represent 95% of total fatty acids (Djemel *et al.*, 2005). The low rates of FA deposition observed during seed development of *M. truncatula*, as well as the high proportion of polyunsaturated FA, seem to reflect ancestral traits of this species which has not been improved for oil production.

### 3.3 Oligosaccharides

In *Medicago*, synthesis of sugars of the Raffinose Family Oligosaccharides (RFO), namely raffinose, stachyose, and verbascose, begins at about mid-maturation, but storage of stachyose, the main RFO stored in *Medicago* occurs mainly during late maturation (see Figure 1), in parallel with a sharp decrease in sucrose content (Djemel *et al.*, 2005). RFO provide an important source of carbon during germination and may also be involved in desiccation tolerance.

Galactomannans play a role as reserve polysaccharides in plants, and are typical of the endosperm of a wide range of legume seeds (Buckeridge *et al.*, 2000). Galactomannans are composed of a linear backbone of β-(1-4)-linked D-mannose residues to which D-galactose residues are attached by β-(1-6)-linkages. In the seeds of fenugreek, crimson clover and lucerne, it has been shown that the aleurone layer of endosperm is non-living and that the cells are almost totally filled with galactomannan (Reid and Meier, 1972). Hydrolysis of these cell wall polysaccharides upon germination helps sustain the embryo. As *M. truncatula* belongs to the same tribe, the Trifoliiaceae, it is likely that galactomannans are also a form of carbon storage in the endosperm.

### 3.4 Minerals

Although constituting less than 3% of the seed dry mass, minerals accumulated during seed development form an important pool of essential nutrients. These minerals are required by the developing seed to enable many cellular processes, but more importantly, are needed to support the growth and early development of the shoot and root tissues of the post-germinative seedling. Several mineral elements are stored in *M. truncatula* seeds, with most believed to be delivered to the seed coat from vegetative source tissues by way of the phloem transport system (Grusak and DellaPenna, 1999). Subsequent distribution of minerals to the embryo would require an apoplastic step, similar to that of sugars and amino acids (Lalonde *et al.*, 2003). Interestingly, the inner epidermis of the pod wall exhibits transfer cell-like wall ingrowths adjacent to the
developing seeds, which might also be involved in the delivery of minerals to the seed coat (Wang and Grusak, 2005).

Mineral concentrations in *M. truncatula* seeds vary amongst ecotypes (Table 1), and most fall within ranges that are similar to those of other legumes. In particular, Fe, Zn and Ca concentrations are elevated, relative to cereal grains, which are a hallmark of legume seeds (Grusak and Cakmak, 2005). Although no data are currently available on the distribution of minerals between the seed coat and embryonic tissues in *M. truncatula*, it is presumed that the majority of the minerals are stored in the cotyledons, which are the largest dry matter component of the seed at maturity. Overall, from the standpoint of seed mineral import and storage, *M. truncatula* appears to provide a good model for the agronomically important legumes that provide dietary minerals to humans and other animals.
Table 1. Seed mineral concentrations found in whole seeds of *Medicago truncatula* and selected agronomic legumes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Macro-elements (mg/g DW)</th>
<th>Micro-elements (µg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca</td>
<td>Mg</td>
</tr>
<tr>
<td><em>Medicago. truncatula</em>¹</td>
<td>1.2 – 2.7</td>
<td>2.6 – 3.2</td>
</tr>
<tr>
<td><em>Pisum sativum</em>²</td>
<td>0.3 – 2.6</td>
<td>1.0 – 2.5</td>
</tr>
<tr>
<td><em>Cicer arietinum</em>³</td>
<td>0.8 – 2.8</td>
<td>1.1 – 2.6</td>
</tr>
<tr>
<td><em>Phaseolus vulgaris</em>⁴</td>
<td>0.6 – 1.8</td>
<td>1.2 – 1.8</td>
</tr>
<tr>
<td><em>Glycine max</em>⁵</td>
<td>1.8 – 3.4</td>
<td>2.2 – 3.1</td>
</tr>
</tbody>
</table>

¹Data from 34 ecotypes grown in a greenhouse using a synthetic soil with all essential minerals provided daily (Grusak, in preparation).
²Data from 481 accessions grown under greenhouse conditions (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?177).
³Data from 197 accessions grown under greenhouse conditions (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?54).
⁴Data from Meiners et al. (1976) and Beebe et al. (2000).
⁵Data from Sale and Campbell (1980) and Raboy et al. (1984).
4 Synthesis and partitioning of metabolites in developing seeds

The accumulation of storage proteins represents a major metabolic event during development of legume seeds and is accompanied by a multitude of changes in gene expression that adapt the seed tissue to assume this role. Besides the structural requirements for accommodating the protein bodies, there is a need to maintain an elevated supply of amino acids and a sustained energy source. These modifications of metabolism in the embryo, but also in the surrounding endosperm and seed coat, are reflected in the changes in patterns of metabolites and gene expression. There are few publications on the subject of metabolites in developing Medicago seeds; most of the results presented hereafter are from Djemel et al., (2005). In this study, the three compartments embryo, endosperm, and seed coat were separated. Only the maturation phase could be analyzed with precision, due to technical difficulties in harvesting separated seed tissue components at very early or late stages. Global approaches, such as transcriptomics and proteomics, were also used to allow the dissection of molecular processes underlying seed development (Firnhaber et al., 2005; Gallardo et al., 2003), and ongoing work is focused on seed tissue-specific expression of genes and response to the changing environment (Gallardo et al., manuscript in prep.). Some results from these transcriptomics and proteomics surveys are presented below along with metabolite profiling.

4.1 Sugar metabolism

At early stages of seed filling, significant but low glucose and fructose concentrations were detected in embryo, endosperm and seed coat compartments. The highest hexose concentrations were found in the endosperm at 10 DAP corresponding to the end of the histodifferentiation phase of embryo development. These concentration values in endosperm were found to vary with ecotypes (from 2% for ecotype Jemalong to 12% for ecotype Salernes). Hexoses never exceeded 3% in 10 DAP seed coat and 1% in the youngest embryos. These concentrations fall sharply in these seed tissues as maturation proceeds (Figures 2 and 4).

In contrast, the sucrose concentration was still high at mid-maturity, then steadily declined. The endosperm and embryo are the most concentrated in sucrose with 15-20% of DW while the seed coat has around 10%. Sucrose concentration decreased rapidly after 13 DAP in the seed coat and the endosperm, reaching only 1-2% values around mid-maturity (19 days) and staying unchanged until maturity. Conversely, the sucrose concentration decreased steadily in the embryo until late maturation. The low amount of sucrose in the seed coat and the endosperm probably represents sucrose originating from phloem unloading transiting to the embryo across the surrounding tissues during the most active period of seed filling.
Figure 4: Carbohydrate composition and DW measurements of the isolated seed coat (B) and endosperm (A) during seed maturation of *M. truncatula*. Data were obtained at least from 3 independent determinations. Broken line represents estimated growth curve for the 2 compartments.

These results reflect an active sugar metabolism in the filling seeds. As concentrations vary between compartments and developmental stages it can be assumed that expression of different enzymes is strongly and differentially regulated. The two key enzymes implicated in the first steps of sucrose metabolism are invertase which converts sucrose to glucose and fructose, and sucrose synthase which converts sucrose to fructose and UDP-Glucose. Invertase is thought to be linked to cell divisions in Fabaceae (Weber *et al.*, 1996), and sucrose synthase is thought to be related to cleavage of sucrose in relation to seed storage (Dejardin *et al.*, 1997). Proteomics revealed a 92 kDa subunit of the tetrameric form of sucrose synthase (MtSucS1 gene) that is highly abundant in embryo cells up to 20 DAP corresponding to the protein deposition phase (Figure 4). Its accumulation correlates with the decrease in the amount of sucrose observed in embryo during maturation (from 19 to 25 DAP, Figure 2). Microarray-based transcriptome analysis also revealed an early and strong induction of sucrose synthase (MtSucS3 gene), as well as expression of an acid invertase gene. In seed coat and endosperm, only degradation products of sucrose synthase (~65 kDa) were recently identified by proteomics at the time of the switch towards storage function, suggesting a turnover for sucrose synthase in these tissues during seed filling (Gallardo *et al.*, in prep).

Altogether, the data reflect a sequential activity of sugar metabolism in embryo and in the surrounding tissues. This agrees with a recent detailed study of seed coat structure that revealed a simple system of vasculature made of a single chalazal vascular bundle with two short branches (Wang and Grusak, 2005) confirming that, as in large seeds, a part of sucrose incoming from maternal tissue can be processed in the seed coat for starch and nitrogen metabolism (Murray 1987; Rochat and Boutin, 1991, see Figure 4).
Figure 5. Protein profiles for embryo-specific sucrose synthase (●, TC100410) and starch synthase (○, TC94194) expressed as log₂(R) during seed development.

R, ratios calculated between values at the stages analysed (14, 16, 20, 24 and 36 DAP) and values at the 12 daf reference stage. R ≥1 (positive log ratios above 2-fold, P value ≤0.05): protein level up-regulated with respect to 12 daf. R ≤−1 (negative log ratios above 2-fold, P value ≤0.05): protein level down-regulated with respect to 12 daf.

4.2 Starch synthesis

Sucrose synthase can play an important role in controlling starch synthesis by supplying UDP glucose as an immediate substrate. In this context, metabolite profiling showed that transient starch storage occurred both in the seed coat and the embryo but not in the endosperm (Figures 2 and 4). Starch represented up to 10% of the seed coat dry weight at early maturation, its concentration decreasing steadily reaching trace amounts from 22 DAP. In the embryo, starch concentration reaches 4-5% by 22 DAP. Proteomics revealed a concomitant accumulation of the enzyme starch synthase that is responsible for starch synthesis in the filial tissue (Figure 5). Starch synthesis was accompanied by an accumulation of proteins related to photosynthesis (e.g. oxygen-evolving enhancer protein and chlorophyll a/b binding proteins) that is thought to furnish oxygen, ATP and NADPH for storage compound synthesis. Interestingly, the level of RuBisCO also increased late in seed development, which may relate to recycling of CO₂ released during this process as it was recently reported for oil seeds such as rapeseed or soybean (Schwender et al., 2004).

The sugar metabolism data are in accordance with the existing models for large-seed Leguminoseae. The seed coat during all the morphogenesis stage is the main component of the seed, its role being to act as a sink for phloem unloading in the small seed which is partly achieved by starch synthesis and temporary storage (Rochat and Boutin, 1992). At this time, a high concentration of hexoses in the endosperm can favor cell division in the embryo (Weber et al., 1996). During maturation, starch in the seed coat is metabolized and the carbon released is furnished to the embryo. The endosperm surrounding the embryo retains a significant level of sucrose consistent with galactomannan synthesis.

4.3 Amino acid metabolism

Free amino acids were abundant in all tissues of the developing seeds at 10 and 13 DAP, reaching concentrations of 10% in seed coats and 20% in endosperms (DW basis), then their concentration decreased sharply and remained very low until the end of maturation. Four free amino acids were especially abundant in the young embryos: glutamine, asparagine, arginine and glutamic acid; these represented about 30% of total amino acids found in mature seeds. A recent transcriptome analysis of seed development based on the use of the Mt16KOLI1 microarrays (K. Gallardo, H. Kuester, C. Firnhaber, et al., manuscript in prep.) revealed a preferential expression of genes encoding enzymes involved in the interconversion of glutamine into glutamic acid, and of asparagine into aspartic acid in seed coat and endosperm tissues. Conversely, genes encoding enzymes involved in arginine synthesis were specifically expressed in embryo. This reflects distinct but coordinated amino acid metabolic activities in
Among metabolites synthesized through amino acid metabolism, several are key players in the regulation of metabolic activities in seeds. This is the case for S-adenosylmethionine (AdoMet) that has an important influence on cell growth and development. Beside its role in ethylene, biotin and polyamine biosynthesis, AdoMet is the primary methyl-group donor for the methylation of amino acids, lipids, RNA and DNA, and functions as an effector in the regulation of threonine, lysine and methionine synthesis (Ravanel et al., 1998). The synthesis of AdoMet from methionine and ATP is catalysed by AdoMet synthetase. In Arabidopsis, AdoMet synthetase was absent from dry mature seeds, but accumulated in the transition from a quiescent to a highly active state during germination (Gallardo et al., 2001). In M. truncatula, a striking decrease in the level of AdoMet synthetase was observed during seed development, that contrarily to germination is characterized by a switch from a period of highly active metabolism associated with cell expansion, differentiation and accumulation of storage products to a period during which the overall biosynthetic activity decreases as the embryo prepares for quiescence.

Following the decrease of AdoMet synthetase, there was an increase in the abundance of enzymes involved in AdoMet consumption. One of them corresponded to AdoHcy (S-Adenosyl homocysteine) hydrolase. The hypothesis that AdoHcy hydrolase is active during seed development agrees with previous results showing that methionine recycling via the AdoMet/AdoHcy and the S-methylmethionine cycles is not sufficient in mature seeds to maintain an appropriate pool of methionine for rapid germination and seedling establishment (Gallardo et al., 2002). The disappearance of AdoMet synthetase and the accumulation of AdoMet consuming enzymes are likely to decrease AdoMet levels. Given the important regulating influence of AdoMet on cell functions, this may promote the repression of the metabolic activities leading to a quiescent state. This suggests that the same mechanism may be implicated in the repression of metabolic activities during seed development and their resumption during germination.

5 Contribution of Maternal Tissues

Seeds require a constant flow of sugars, amino acids, and mineral ions to support their growth and development. These solutes come from various maternal source tissues where they are synthesized, absorbed, and/or accumulated before being delivered to the seeds. Although specific data on source-sink dynamics are limited in M. truncatula, studies with M. sativa and other legumes suggest that all maternal tissues in M. truncatula are contributing to seed growth in some way. These tissues would include leaflets, stems, and pod walls as primary regions where solutes are phloem-loaded for export to the seeds. Seed coats (maternal tissue) also would be involved in nutrient flow, either as a pass-through tissue or for the metabolism of certain nutrients, before their apoplastic release to the embryo. Finally, roots can be viewed as secondary source regions, in that they absorb minerals and metabolize amino acids that are delivered to leaves, stems, and pod walls via the xylem pathway.

5.1 Delivery of sugars

Photosynthetically competent leaflets, stems, and pod walls are all potential donors of sugars to M. truncatula seeds; they can mobilize both recently fixed photoassimilates as well as previously stored carbohydrates. Leaves are undoubtedly an important source of these sugars because of their high rate of photosynthesis (relative to stems and pod walls), and because defoliation at flowering has been shown to reduce specific seed weight in M. sativa (Genter et al., 1997). Furthermore, because seed growth is not completely halted upon defoliation, this implies that stems and pod walls must be responsible for some of the carbohydrates transported to developing seeds. In fact, stem and petiole dry weight has been shown to
decline in *M. truncatula* during reproductive development (Alston and Puckridge, 1986; Derkaoui et al., 1990). In *M. truncatula* pod walls, on the other hand, relatively few starch grains are present in mesocarp cells at 20 DAP (Wang and Grusak, 2005), suggesting that there is little reserve carbohydrate for partitioning to seeds. Furthermore, pod wall dry weight does not decrease with maturity in *M. truncatula* (Alston and Puckridge, 1986; Grusak, unpublished results), unlike what is seen in some other legumes (Harvey et al., 1976); thus, there does not appear to be a net turnover of stored dry matter from pod walls to seeds.

Seed coats in *M. truncatula* are presumably photosynthetically competent, because they contain chlorophyll until late in development. Seed coats in other legumes have been shown to be capable of refixing respired CO$_2$ that accumulates in the pod cavity (Willmer and Johnston, 1976). This may also occur in *M. truncatula*, but it is not known how much respired CO$_2$ might be fixed by the seed coat, or what percent of this might be partitioned to the embryo.

5.2 **Delivery of amino acids**

As with sugars, several maternal organs are involved in the delivery of amino acids to developing seeds, with these organs drawing upon either recently acquired amino acids or those recovered from stored proteins. Most source organs (e.g., stems, leaves, and pod walls) are believed to load amino acids into the phloem pathway (Lalonde et al., 2003). Similar loading capacity is anticipated to occur in *M. truncatula*. These amino acids come from nitrogen acquired by the roots (e.g., as nitrate, ammonium, or N$_2$ fixation by root nodules) and are synthesized predominantly in the roots. They then are delivered to above-ground vegetative tissues via xylem flow. Organs with high rates of transpiration (e.g., leaves) will thus acquire the bulk of these amino acids, and are probably the primary source region for remobilization of these newly fixed nutrients. Nitrogen absorbed during reproductive growth contributes significantly to seed protein deposition in pea (Schiltz et al., 2005).

Long-term storage of nitrogen also occurs, with vegetative storage proteins (VSPs) having been described in several species (Staswick, 1994). In addition, structural proteins, such as Rubisco, can be degraded for amino acid mobilization to the seeds when vegetative organs senesce (Jiang et al., 1993). Studies with *M. truncatula* show that nitrogen turnover occurs in leaves, stems and pod walls (Alston and Puckridge, 1986), although it is unclear whether the turnover is due to VSPs, structural proteins, or both. In soybean leaves, the bulk of the VSPs are accumulated in the paraveinal mesophyll, a specialized region of the leaf (Franceschi and Giaquinta, 1983). This structure has not been identified in leaves of *M. truncatula*, although an attenuated paraveinal mesophyll has been noted in *M. sativa* (Kevekordes et al. 1988).

5.3 **Delivery of minerals**

As with the amino acids, all maternal organs play some role in the delivery of mineral ions to the seeds. Minerals enter the plant via the root system and are subsequently supplied to transpiring shoot organs via the xylem pathway. Once in leaves, stems, and pod walls, minerals are loaded into the phloem transport stream for partitioning to seeds. Little information is available on mineral turnover in maternal tissues of *M. truncatula*, but it is likely that all shoot tissues can load minerals into the phloem (e.g., as with Fe in *Pisum sativum*; Grusak, 1994). It also appears that for most minerals, continued root uptake is needed throughout reproductive growth to supply the shoot source regions with mineral substrates for redistribution (Hocking and Pate, 1977).
6 Maturation and Acquisition of Desiccation Tolerance

During seed development of *M. truncatula*, embryogenesis is completed around 10-12 days after pollination (DAP), whereas seeds are mature around 28-36 DAP. In between, the biochemical and physiological changes that take place are mainly related to reserve accumulation, acquisition of germination capacity, desiccation tolerance, maturation drying and the installation of dormancy (Fig. 1). While these processes are well documented for many legume species, the picture emerging for *M. truncatula* is still sketchy but appears to depend both on the genotype and growth conditions.

![Image of physiological events](image)

**Figure 6. Main physiological events occurring during maturation of *M. truncatula* seeds.** Data are shown for genotype Paraggio, and resemble those of genotype Jemalong. Acquisition of precocious germination was determined by imbibing 50-75 seeds right after excision form the pods, whereas desiccation tolerance was determined by first drying 50-75 seeds fast under an air flow of 43% RH. Fresh and dry weight was determined gravimetrically on three times four seeds.

Between ca. 12-14 DAP, seeds of *M. truncatula* gain the capacity to germinate if removed from the pod and imbibed on wet filter paper (Fig. 1 in Gallardo *et al.*, 2003). Thereafter, the rate of germination increases and peaks at around 26 DAP (Gallardo *et al.*, 2003). Desiccation tolerance corresponds to the capacity to withstand an enforced removal of most of the cellular water (e.g. below 0.07 g H2O/g dw). For seeds that are dried rapidly under an airflow at 43% relative humidity (i.e. within several hours), desiccation tolerance is acquired two to three days later than precocious germination (16-19 DAP) (Fig. 1). As for other species, slow drying of the immature seeds (i.e. within several days) leads to further maturation. By this way desiccation tolerance appears concomitantly with the germination capacity (Gallardo *et al.*, 2003). As for other species, desiccation tolerance is acquired first by the cotyledons and then by the embryonic axes, albeit with less then 12-24h difference between these organs. At 20 DAP, seeds are fully capable of surviving rapid drying and sustaining prolonged storage in the dry state (Fig. 6; Boudet *et al.*, 2006, Buitink *et al.*, 2006). At this age, they are midway through the filling processes (see 3.) and the seed coat is still green. A transcriptomic analysis of desiccation-tolerant 20 days-old seeds compared to sensitive seeds that were harvested at 14 DAP revealed large shifts in the transcriptome (Buitink *et al.*, 2006; Firnhaber *et al.*, 2005). For example, up-regulated genes belong to classes that are related to protective functions in the dry state, such as late embryogenesis abundant (LEA) proteins, small heat shock proteins,
and antioxidants, as well as genes encoding proteins involved in several regulatory functions, such as transcription factors, calcineurin B-like proteins and snf4b, a putative activating subunit of the sucrose nonfermenting related kinase (SnRK1) complex. In parallel, a massive down-regulation takes place of many genes involved in primary metabolism, cell cycle and biogenesis (Buitink et al., 2006; Firnhaber et al., 2005).

At the end of the maturation, oligosaccharides are accumulating, with stachyose becoming the most prominent non-reducing sugar around 28 DAP (Djemel et al., 2005). From this point on, dry matter of the embryo ceases to accumulate, maturation drying is initiated and the seed coat looses its chlorophyll (Fig. 7; Djemel, 2005), although it appears that different growth conditions can shift the initiation of maturation drying towards 36 DAP (Gallardo et al., 2003). Mature *M. truncatula* seeds exhibit at least two types of seed coat-enhanced dormancy (Faria et al., 2005). Physiological dormancy appears to be acquired between 30 and 40 DAP, according to the genotype, whereas physical dormancy, i.e. seed hardiness, is induced by the maturation drying (see 7.). At these later stages of maturation, the longevity of orthodox seeds is known to increase and depends on the temperature and water content of storage (Buitink et al., 2000). Although the shelf life of *M. truncatula* seeds under ambient conditions has not yet been determined, it is likely to be high compared to seeds of other species. For example, seeds of the Salernes genotype harvested in 1995 still exhibit 80% of germination in 2006 when stored under ambient conditions (Buitink, unpublished data). As a comparison, the half viability period both of *M. lupulina* and *M. sativa* is estimated at 9-11 years when stored under similar conditions (Priestley et al., 1985). Measurements of seed longevity of several genotypes indicate that the storability, when measured under accelerated ageing conditions (45°C, 75% RH) is different among genotypes (J. Buitink, unpublished data).

Desiccation tolerance and longevity are gradually lost during seed imbibition, first in the germinated radicles, then in the cotyledons. Even so, it is possible to re-establish desiccation tolerance in emerged radicles by incubating germinated seeds for several days in an osmotic solution at -1.7 MPa (Buitink *et al.*, 2003). Transcriptome analysis shows that a number of genes expressed specifically in maturating seeds are re-induced in these radicles. Likewise, many genes that are down-regulated at the later maturation phase are also repressed in the later phase of osmotic treatment in the germinated radicles that have become desiccation tolerant (Buitink *et al.*, 2006). Therefore, there might exist a developmental window during germination, which allows a return to a maturation program upon a small loss of water, as demonstrated for *Arabidopsis thaliana*.

7 Dormancy and Germination

Seeds of wild species such as those of *M. truncatula* often show stronger dormancy than cultivated genotypes. Seed dormancy is defined as the (temporary) failure of an intact viable seed to germinate under otherwise favourable conditions. Dormancy is regulated by many genes and depends strongly on environmental factors, making it a typical quantitative trait. At seed dispersal, two types of primary dormancy are encountered that are both seed-coat-imposed. Physical seed-coat imposed dormancy corresponds to seed hardiness, a known characteristic for legume seeds that impedes or severely slows down the imbibition process, thereby inhibiting radicle growth. Seed hardiness in *M. truncatula* may vary from 10 to 100% within a seed lot, depending on the genotype and environmental conditions (Taylor, 1996). Like many other legume species, seed hardiness is influenced by seasonal factors: it is low in spring and very high in other seasons, particularly when temperature fluctuations follow a chilling period (Taylor, 1996; Van Assche *et al.*, 2003). This type of dormancy can be conveniently removed by physically damaging the seed coat by rubbing the seeds on sand paper (i.e. scarification, van Assche *et al.* 2003) or chemically by incubating the seeds in
The second type of dormancy is physiological; even when seeds imbibe fully they will not germinate or do so very slowly (within several weeks to months, depending on the genotype and germination temperature). Removal of the seed coat/endosperm from the dormant seeds restores their germination capacity, indicating that it is the seed coat or more likely the endosperm that prevents the germination. Dormant seeds need to be exposed to warm-dry conditions for several weeks/months (after-ripening) for the dormancy to be gradually released. For example, in the genotype Jemalong, the germination percentage after 1 month of harvest was 40%, but increased to 86% after 6 months of after-ripening. This type of dormancy can be released by imbibition in the cold (4°C) (Faria et al., 2005). Characteristically, dormancy can also be re-induced in non-dormant germinating seeds by unfavourable conditions, and is referred as secondary dormancy. However, it is unknown whether this phenomenon can occur in M. truncatula seeds.

Once dormancy is released, seeds imbibe rapidly and germinate readily by placing them simply on wet filter paper. This way, germination, which encompasses those events starting with the imbibition and ending with the visible emergence of the radicle, occurs within one to two days both in the dark and in the light, the speed of which depends on the genotype and temperature (see Buitink et al., 2003; Faria et al., 2005). The optimal temperature for germination is around 20-22°C, depending on the genotype. At these temperatures, 50 % of radicle emergence is obtained with 19-22 h. A third factor that influences the germination rate is the external water potential of the solution. When seeds are exposed to osmotic solutions of around -1 to -1.5 MPa, the restricted water content will not lead to radicle protrusion, although some events linked to germination do occur during this period. As for crop seeds, this exposure to low water potentials (known as seed priming; McDonald, 2000) considerably increases the speed of germination of the re-dried seeds. Priming of M. truncatula seeds for several days can enhance germination rate of re-dried seeds over three-fold, leading to germination of half of the seed population within 7-8h.

During both germination sensu stricto and early post-germinative growth, seedlings are heterotrophic relying only on seed reserves. Upon imbibition of Medicago truncatula seeds, nitrogen and carbon metabolism resume at very early stages of germination as revealed by changes in amino acids and soluble sugar content (Fig. 7; see Glevarec et al. 2004). Comparison of composition of free amino acids pool with that of protein amino acids indicated that high amino acid inter-conversion takes place in cotyledons after protein degradation and in embryo axis after amino acid import. Total amount of free amino acids in the dry seed was 1413 nmoles and was represented essentially (62%) by Arg, Tyr and Asn (Fig. 7a). At 21h of imbibition, in darkness at 19°C, right before radicle emergence, cotyledons and embryo axis contained respectively 1864 and 665 nmoles, respectively that represented a 1.7 fold increase in total seed amino acids compared to the dry seed. After radicle emergence, total amount of free amino acids sharply increased and at 48h cotyledons and embryo axis contained respectively 5404 and 13006 nmoles that represented a 13 fold increase in total seed amino acids compared to the dry seed (Fig. 7a). Major amino acids in cotyledons were Trp, Asn, His and Arg representing 65% of total amino acids whereas in the embryo axis, the majority of amino acids (66%) was accounted for by Asn, His, Ala and Ser. The amount of ammonium was low and stable in the early stages of germination and increased sharply after radicle emergence essentially in the embryo axis (Fig. 7a) (Glevarec et al., 2004). Amounts of glucose and fructose were very low before radicle emergence (Fig. 7c). Afterwards they remained at a hardly detectable level in cotyledons whereas they increased sharply in the embryo axes. The amount of sucrose increased significantly in the embryo axis throughout germination. In cotyledons, the amount of sucrose increased dramatically in
the first hours of imbibition up to 15 hours, remained constant during post-germinative growth and dropped at 48 hours indicating the exhaustion of carbon (starch) reserves. The amount of citrate decreased in cotyledons during the first hours of imbibition, then remained nearly constant throughout germination. In embryo axis, amount of citrate increased steadily in late stages of germination to reach almost the same amount as in cotyledons at 48 hours. Oxaloacetate was detected only in cotyledons where its amount remained stable throughout germination. Malate and 2-oxoglutarate were detected neither in cotyledons nor in embryo axis (Fig. 7c) (Glevarec et al., 2004).

In germinating *Medicago truncatula* seeds, it was found that expression of *GS1b* (glutamine synthetase) was about 10-fold higher than that of *GS1a* that was expressed at a low level. This finding suggests that GS1b is very probably the isoform responsible for re-assimilation of ammonium released from protein and amino acid catabolism. Expression of NADH-dependent *GOGAT* (glutamate synthase) was predominant, compared to Fd-dependent isoform essentially in the embryo axis where it showed an important increase during post-germinative growth when amino acid synthesis was at its utmost. Expression of chloroplastic *GS2* and Fd-dependent *GOGAT* increased significantly in cotyledons but not in embryo axis during post-germinative growth. Taken together these results show that the cytosolic GS1b/NADH-GOGAT cycle constitutes the major route of assimilation of ammonium derived from reserve mobilization and Glu/Gln synthesis in heterotrophic seedlings of *Medicago truncatula* during germination *sensu stricto* and in the embryo axis during post-germinative growth maintained in darkness and in the absence of an exogenous source of nitrogen (NO₃⁻). In cotyledons however, during post-germinative growth the major route of ammonium assimilation is constituted by the couple GS2 / Fd-GOGAT.

Two major connecting points between carbon and nitrogen metabolism are the reactions catalyzed by PEPc and I(C)DH that generate carbon skeletons readily used for amino acid synthesis. Accumulation of oxaloacetate in cotyledons but not in embryo axis suggests the importance of the anapleurotic role of PEPc in the generation of asparagine, the AA transported in legumes. Expression of the cytosolic *cICDH* gene was two to four times higher than that of the mitochondrial *mIDH* in both embryo axis and cotyledons providing an argument in favor of ICDH being the provider of 2-oxoglutarate for amino acids synthesis. This result agrees with the idea that ICDH is rather involved in metabolic functions related to Glu / Gln production for import / export into different tissues (Lancien et al., 2000). Among the metabolic enzymes induced during germination, the one that GDH (glutamate dehydrogenase) showed the highest increase, up to 80-fold in gene expression (Fig. 8), accompanied by a consistent increase in the amount of GDH-protein in the embryo axis during post-germinative growth. This increase was concomitant to the sharp increase in free ammonium in the embryo axis (Fig. 7a). ¹⁵NH₄ labeling experiments allowed to rule out any contribution of GDH to ammonium assimilation. The hypothesis of an anapleurotic role as 2-oxoglutarate provider suffers a lack of coherence since in terms of balance the carbon skeleton produced by glutamate catabolism would be consumed by the assimilation of ammonium released during the same reaction. Furthermore, two enzymes that funnel carbon skeletons to nitrogen metabolism are active in the germinating seed *i.e.* PEPc and I(C)DH. The only consistent hypothesis is the one that attributes a catabolic role to GDH in the embryo axis. Catabolism of Glu by GDH being one of the major sources of ammonium in seeds for glutamine synthesis by GS in the absence of primary nitrogen (NO₃⁻) assimilation. Furthermore, this reaction produces reducing power (NADH) in heterotrophic seedlings deprived of photosynthesis (Glevarec et al., 2004).
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