A combined histology and transcriptome analysis unravels novel questions on *Medicago truncatula* seed coat

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Abstract

The seed coat is involved in the determination of seed quality traits such as seed size, seed composition, seed permeability, and hormonal regulation. Understanding seed coat structure is therefore a prerequisite to deciphering the genetic mechanisms that govern seed coat functions. By combining histological and transcriptomic data analyses, cellular and molecular events occurring during *Medicago truncatula* seed coat development were dissected in order to relate structure to function and pinpoint target genes potentially involved in seed coat traits controlling final seed quality traits. The analyses revealed the complexity of the seed coat transcriptome, which contains >30 000 genes. In parallel, a set of genes showing a preferential expression in seed coat that may be involved in more specific functions was identified. The study describes how seed coat anatomy and morphological changes affect final seed quality such as seed size, seed composition, seed permeability, and hormonal regulation. Putative regulator genes of different processes have been identified as potential candidates for further functional genomic studies to improve agronomical seed traits. The study also raises new questions concerning the implication of seed coat endopolyploidy in cell expansion and the participation of the seed coat in *de novo* abscisic acid biosynthesis at early seed filling.

Key words: Abscisic acid, endopolyploidy, *Medicago truncatula*, seed coat, structure and ultrastructure, transcriptomic profiling.

Introduction

After fertilization, differentiation of the ovule integuments to form the seed coat provides the embryo with an interface with the mother plant. Seed coat differentiation serves as a protective barrier to the embryo, but also as the transient storage tissue which delivers nutrients from the mother plant. Genetic and molecular analyses in *Arabidopsis* have shown the effect of the seed coat on many aspects of seed biology, including seed development, seed size, and shape (Leon-Kloosterziel et al., 1994; Debeaujon et al., 2003; Garcia et al., 2005; Haughn and Chaudhury; 2005). The *transparent testa* genes, involved in regulation of epidermal morphogenesis, indicated that the control of seed germination and dormancy is linked to seed coat structure (Debeaujon et al., 2000; Tsujiya et al., 2004; Dean et al., 2011). Biochemical and transcriptomic studies highlighted regulation of the biosynthesis of flavonoids and mucilage (Western et al., 2001, 2004; Lepiniec et al., 2006; reviewed in North et al., 2010).

In grain legumes, mutations altering seed coat structure and functions involved in the control of nutrient flow affect embryo and endosperm development (Patrick and Offler, 2001). The seed coat controls the ratio of hexose to sucrose delivered to the embryo via the associated invertases, sucrose synthase, and sucrose transporters (Dejardin et al., 1997; reviewed in Weber et al., 2005), which indicates the key role of the seed coat during seed development. The occurrence of a large number of seed coat-specific genes, including chalcone synthases and peroxidases, was confirmed by microarray analyses in soybean (Ranathunge et al., 2010).
In the model legume *Medicago truncatula*, genomic and genetic resources may facilitate functional characterization of seed coat genes that could potentially be used in crop legume improvement (Domoney et al., 2006; Young et al., 2011). Transcript and protein distributions in the different seed compartments confirmed the contributions of the seed coat to embryo nutrition and highlighted the persistence of several proteases in the seed coat, showing the importance of proteolysis as a supplementary source of amino acids (Gallardo et al., 2007). The cellular events underlying seed coat differentiation in *M. truncatula* have received little attention. The structure of the *M. truncatula* seed coat corresponds to a typical legume seed coat including macrosclerids, osteosclerids in the outer integument, and parenchyma with endothelium in the inner integument (Wang and Grusak, 2005; Gallardo et al., 2006). In their study, Wang and Grusak focused on the characterization of the pod wall and seed coat as two structural attributes of the maternal reproductive tissues.

In order to relate function to structure, in this study the seed coat transcriptome was analysed at key stages of seed coat development to identify possible target genes controlling final seed traits. Analyses were also performed using light and electron microscopy to reveal the morphological changes in relation to the transcriptomic data. The histological modifications in the seed coat were associated with embryo maturation. They involved cell expansion with the probable initiation of endopolyploidy, cuticle formation, and cell wall thickenings in the outer integuments, which interfere with seed biology and the regulation of seed permeability. Immunolocalization of abscisic acid (ABA) in the plastids of parenchyma cells at early seed filling indicated that this endogenous ABA could be involved in the switch to the maturation stage and seed filling. Biosynthesis of ABA in the seed coat at early seed maturation and the implication of endopolyploidy in cell expansion are discussed using a combination of histological analysis and transcriptomic profiling. The data highlighted how the seed coat may impact seed size, seed composition, and seed permeability, enabling new potential regulator genes to be proposed.

**Materials and methods**

**Plant material**

Immature *M. truncatula* (Jemalong A17) seeds were harvested from plants growing in growth chambers at 22/19 °C (day/night). The different development stages analysed were 4, 6, 8, 12, 14, 16, and 20 days after pollination (DAP), corresponding to embryogenesis and early maturation phases when the embryo goes through globular to late torpedo/bent cotyledon stages.

**Cytological procedures**

Seed were fixed for 24 h with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C. Triton X-100 (1%) was added to the specimens used for immunodetection. After removal of fixative using PBS solution, the samples were dehydrated with a graded ethanol series. Seeds were then infiltrated and embedded with a low viscosity acrylic resin LRWhite (London Resin Company). Sections (2–3 µm) were used for immunolocalization and histological studies. Seed sections at different developmental stages were stained using 1% toluidine blue in sodium carbonate solution (pH 7). Toluidine blue was also used to detect polysaccharides as a metachromatic stain. Toluidine blue develops a greenish-blue colour when associated with polyphenolic compounds such as proanthocyanidins and lignins. It also stains pectins and pectic substances in pink and nucleic acids/proteins in purple.

Fluorescent 4',6-diamidino-2-phenylindole (DAPI; 1 µg ml⁻¹) in Mc Ilvaine buffer (pH 5.5) was used to visualize nuclear DNA. Image analysis of DAPI-stained nuclei was carried out using VISILOG software (Noesis, France). An application was developed to measure fluorescence intensity with the size of nuclei. The amount of fluorescence was divided by the size of the nucleus, which corresponds to the integrated fluorescence. Volume differences were therefore controlled when comparing fluorescence intensities. Two internal controls were tested: the diploid 2n embryo nuclei and the polyploid endosperm nuclei ≥3n, showing the reliability of the measurements. DAPI-integrated nuclear fluorescence was assessed statistically with the Kruskal–Wallis test and visually compared with strip charts (R, version 2.11.1, R Development Core Team, 2010).

For electron microscope observations, ultrathin sections were stained with uranyl acetate and lead citrate, then analysed using a transmission electron microscope (Hitachi H7500). Cell wall thickness was measured on 50 electron microscope micrographs using an application in VISILOG software. Scanning electron microscope observations of mature A17 seeds required 1.5 min metallization with gold–palladium, which was carried out in a high vacuum with a XL30 Philips microscope. For Nomarski microscopy, seeds of 6–10 DAP were fixed for 1 h in ethanol:acid (3:1). Clearing was obtained after incubation in chloral hydrate:water:glycerol (3:8:4,w/v/v) for 3 h. Microscopic observations were performed with Nomarski differential interference contrast (DIC) optics.

**Immunolocalization protocol**

The seed sections (2–3 µm) were collected on slides treated with Dako, then rinsed with PBS containing 1% Tween-20. Before incubation with monoclonal antibody MAC252 anti-ABA (Quarrie et al., 1988; Barrieu and Simonneau, 2000), sections were immersed for 30 min in PBS solution containing 5% bovine serum albumin (BSA) to reduce unspecific binding and incubated for 1 h at 37 °C. They were pre-stained with toluidine blue to prevent autofluorescence. After washing with PBS/Tween-20, sections were incubated with the secondary antibody, a goat antimouse IgG conjugated to the green Alexa 488 fluorochrome, for 1 h at 37 °C. Control sections were only incubated with the secondary antibody. Finally, slides were washed with PBS pH 7.2, then mounted with vectashield mounting medium (Vector Labs) containing propidium iodide for counterstaining. Sections were analysed with an epifluorescence microscope Leica DMRB. Phosphate buffer used at pH 7.2 helps to stabilize and immobilize ABA in the chloroplasts (as revealed by Milborrow, 2001). Autofluorescence, which may interfere with the specific signal, was not observed; the negative control sections did not show any fluorescence.
Transcriptomic analysis

Transcriptomic data, publicly available in the *M. truncatula* Gene Expression Atlas (MtGEA; He et al., 2009) were used. The transcriptomic data sets generated at the Noble Foundation were downloaded and combined, using the Affymetrix GeneChip Medicago Genome arrays containing 50 900 probe sets (i.e. a collection of probes hybridizing to a specific gene or a gene family) and available in the Gene Expression Atlas (www.mtgea.noble.org/v2/). The temporal expression data set, which contains six stages of seed development (Benedito et al., 2008), was combined with a spatial expression data set containing probe sets expressed in seed coat (Pang et al., 2008). In order to allow comparison, these data sets were normalized using the robust multiarray average method for global normalization (RMA; Irizarry et al., 2003). Presence/absence data for each probe set were obtained using dCHIP to discriminate expressed probe sets from background noise (Li and Wong, 2001). Then, the normalized gene expression was analysed using the FOM algorithm to determine the optimal number of clusters and classified by performing a k-mean clustering analysis using Pearson correlation with the Multi Experiment Viewer 4 software (MeV 4). Functional classification of probe sets according to the KEGG ontology was carried out using GeneBins (Ogata et al., 1999; Goffard and Weiller, 2007) to identify over-represented classes for each of the four clusters.

GeneBins calculated a *P*-value using the hypergeometric distribution (http://bioinfolserver.rsbs.anu.edu.au/utils/GeneBins/). This *P*-value represents the probability that the intersection of the submitted probe sets with the set of sequences belonging to this class occurs by chance (Goffard and Weiller, 2007). A significant over-representation of a class was considered when the *P*-value threshold was <5%, with a Bonferroni correction.

Results and Discussion

**Transcriptomic analysis revealed a complex gene regulation of seed coat development**

In this study, a time course of genes expressed in seed coat from 10 to 36 DAP was generated from publicly available transcriptomic data in *M. truncatula*. Two data sets were combined, one corresponding to a temporal study of the whole seed development (i.e. seeds containing seed coat, endosperm, and embryo) (Benedito et al., 2008), and the other corresponding to a spatial expression study revealing probe sets expressed specifically in the seed coat (Pang et al., 2008). By merging these two experiments, expression profiles of probe sets in the seed coat at the different time points described previously were revealed and all probe sets without seed coat expression were discarded. A total of 30 732 probe sets expressed in seed coat were identified, including 1505 with preferential seed coat expression (i.e. expressed 2-fold more in the seed coat than in the whole seed), which indicates that the majority of the seed coat genes were also expressed in other seed tissues. In Supplementary Table S1 available at JXB online, all probe sets expressed in the seed coat are presented and those with preferential seed coat expression are highlighted in yellow. Out of these 30 732 probe sets expressed in the seed coat, the expression values of 30 001 probe sets were detected throughout seed development (Supplementary Table S1). The remaining 731 probe sets were identified as expressed in seed coat from Pang et al. (2008), but without any detectable expression in the whole seed data set from Benedito et al. (2008), probably due to their low expression levels. The list of these 731 probe sets is also included in Supplementary Table S1.

A k-means clustering analysis of the 30 001 probe sets was performed and they were classified into four major clusters during seed coat development according to their temporal expression; this clustering reflects the global seed development (Supplementary Fig. S1 and Table S2 at JXB online). At late embryogenesis, cluster I, which was made up of 9431 probe sets displaying a peak of expression at 10 DAP, corresponds to early seed coat development. Clusters II (of 5573 probe sets that peak at 16 DAP) and III (of 5315 probe sets peaking at 20–24 DAP) correspond to mid seed coat development and seed desiccation. Interestingly, the same cluster pattern was identified throughout the same time course of seed development from *Medicago* whole seeds in two different studies: from 39 194 Affymetrix probe sets differentially expressed in the three seed tissues (Verdier et al., 2012) and from 700 transcription factor expression profiles analysed by qRT-PCR in whole seeds (Verdier et al., 2008). The similar clustering structure between the whole seed transcriptome and the seed coat transcriptome is probably due to a large proportion of probe sets (30 001/39 194) expressed in both seed coat and other seed tissues. This result highlights the complexity of the seed coat transcriptome, consisting of expression of 30 001 probe sets during seed development, which represents up to 76.5% of the whole seed transcriptome complexity (i.e. 30 001 probe sets expressed in seed coat with respect to 39 194 probe sets expressed in whole seed). Such complexity has also been reported in soybean (Ranathunge et al., 2010). Moreover, the 1505 probe sets showing a preferential seed coat expression correspond to genes probably involved in specific seed coat functions. They represent putative target genes to improve seed traits such as seed size, seed composition, seed permeability, and hormonal regulation.

**New insight into cell expansion during early seed coat development**

Growth of the seed coat is mainly regulated by genetic interactions between maternal and filial tissues that ultimately establish seed size. In *Arabidopsis*, the maternally expressed transcription factor *TTG2* (TRANSPARENT TESTA GLABRA2) was shown to be involved in the regulation of cell elongation in the seed coat, and the restriction of cell elongation in the *ttg2* mutants limits endosperm growth. Conversely, in *haiku* (*iku*) mutants, the defect in endosperm growth affects cell elongation in the seed coat with the production of seeds of reduced size (Garcia et al., 2005; Haugn and Chaudhury, 2005). Other factors controlling the number of integument cells, such as the AUXIN RESPONSE FACTOR (ARF2) gene, were also found to impact seed size (Schruff et al., 2006).
Seed coat differentiation, with the formation of outer and inner integuments, in *M. truncatula* 4 DAP is shown in Fig. 1A. At early seed development, the anatomy of the seed coat and its thickness were uniform, the palisade epidermis being the most prominent (Fig. 1B–D). The arrest of cell division, which occurred 4–6 DAP, is compensated for by cell elongation in the expanding seed coat during seed development. The ploidy status of the seed coat nuclei was checked to determine if there is any correlation with cell elongation, endopolyploidy being one of the well-documented factors controlling organ size (Kondorosi et al., 2000).

DAPI-stained seed sections were analysed at the torpedo embryo stage (8–10 DAP). The DAPI-integrated fluorescence, which correlates to DNA quantity, was measured in the three seed tissues (Fig. 1E–H; Table 1). Fluorescence values were compared in the seed coat parenchyma nuclei and in the diploid embryo nuclei used as internal control. Significantly higher values were obtained in the seed coat nuclei, suggesting a higher DNA content (Fig. 2). The fluorescence values of seed coat nuclei were

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<th>Mean</th>
<th>Minimum</th>
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<tr>
<td>Embryo</td>
<td>18 020</td>
<td>10 830</td>
<td>34 450</td>
<td>4631</td>
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<td>Testa</td>
<td>26 240</td>
<td>7 950</td>
<td>56 700</td>
<td>8 349</td>
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<tr>
<td>Endosperm</td>
<td>85 510</td>
<td>33 540</td>
<td>195 900</td>
<td>40 171</td>
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* Endosperm cells are the endopolyploidy control 2x ≥ 3n.
Fig. 2. Distribution of the DAPI integrated intensity in the diploid embryo nuclei and the testa nuclei. The integrated intensity was shown to be significantly different between the two cell types: Welch two-sample t-test, \( t=9.66, \text{df}=219, P < 0.001 \).

between those of the two internal controls, namely the diploid embryo nuclei \( 2n=2x \) and the endopolyploid endosperm nuclei \( 2n \geq 3x \). In the analysed nuclei, the statistical analysis showed three different ploidy levels (Supplementary Fig. S2 at \( JXB \) online).

At this stage, cell divisions were not detected in the seed coat, but cell elongation was initiated and may be correlated to the induction of endopolyploidy (Supplementary Fig. S2). The correlation between the nuclear DNA content and cell volumes has been established during eukaryotic evolution (Cavalier-Smith, 2005). In plants, endopolyploidy was implicated in cell size control, and the polyploid nuclei were associated with enlarged cell size via an increase in cell growth (Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003; Lee et al., 2004). The growth of tomato fruit was characterized by the occurrence of numerous rounds of DNA endoreduplication in connection with cell expansion and final fruit size determination (Mathieu-Rivet et al., 2010). Finally, in transgenic plants, additional versus reduced rounds of endoreduplication were correlated with cell and organ size (Castellano et al., 2004; Vliegh et al., 2005). Even if the positive correlation between ploidy level and cell size in \( M. \) truncatula seed coat needs to be validated, little contradictory evidence is known. Moreover, it opens up a new perspective to control final seed size.

According to the transcriptomic analysis (KEGG annotation and GeneBins), cluster I, at late embryogenesis, revealed an overexpression of genes related to the ‘nucleotide metabolism’ class, with a maximum expression at 10 DAP (Fig 3; Supplementary Table S2 at \( JXB \) online). Both ‘purine and pyrimidine metabolism’ classes were over-represented in this cluster. For instance, out of the total 32 probe sets present in the Affymetrix chip, 18 probe sets related to ‘DNA polymerase’ class were present at this stage (e.g. Mtr.9433.1.S1_at, Mtr.49500.1.S1_at, and Mtr.8328.1.S1_at). With cell division being absent in the seed coat at this stage, the high proportion of genes annotated as involved in nucleotide metabolism supports the initiation of endocycles.

The detection of a high level of RCC1 transcripts (REGULATOR OF CHROMOSOME CONDENSATION), essential for DNA replication, and other transcripts such as 8-TRANSUDIN transcripts related to the family of WD-40 genes implicated in the setup of endocycles in \( Drosophila \) and plants, is in agreement with this hypothesis (Supplementary Table S1).

Mid seed coat development, embryo nutrition, and final seed composition

At the bent cotyledon stage, the onset of seed filling (12 DAP), the seed coat structure showed a switch to specialized cell types including differentiation of the endothelium layer (Fig. 4B, C). In the innermost cell layer of the seed coat, the endothelium displayed characteristics of high metabolic cell activity such as dense cytoplasm with small vacuoles, abundant mitochondria, and the presence of plasmodesmata through the thin cell wall promoting intercellular exchange.

The unique phloem vein, consisting of three unequal branches, was rooted in the parenchyma of the micropylar–chalazal extension region where the seed coat was more enlarged (Fig. 4A, D, G). Xylem bundles were also detected at the hilum (Fig. 4E, F). The organization of the vascular bundles may have an effect on the passive nutrient efflux through the seed coat. The flow of nutrients which control embryo nutrition passes through the seed coat via the phloem, which controls the sugar status and the amino acid efflux (Patrick and Offler, 2001; reviewed in Weber et al., 2005). In grain legumes, the vascular network has also served as a useful characteristic for taxonomy. A single chalazal
Fig. 3. Functional classification of the 30,001 genes expressed in the seed coat, according to the Kyoto Encyclopedia of Genes and Genomes, in four major clusters (clusters I, II, III, and IV, respectively, from left to right). Using GeneBins, significant over-representation of a class (P-value < 5% with Bonferroni correction) is indicated with plain bars (white bars represent classes which are not statistically over-represented).

Fig. 4. The Medicago truncatula seed sections 12 DAP prior to seed filling in light microscopy. Seed sections are stained with toluidine blue. (A) The single chalazal vein (vb) with three unequal branches is shown. (B and C) The structure of the seed coat as observed at the chalazal–micropyle region where it is much larger (B), and outside this region (C). The differentiation of the endothelium (ed), in the inner integument, was adjacent to the vein was present in other members of the Viciae tribe, while the Phaseoleae tribe was distinguished by an extensive seed coat vascular network (van Dongen et al., 2003).

As shown in Fig. 4G and H, the branched parenchyma cells associated with the vascular bundle were much larger than their neighbours, and the presence of intercellular spaces allowed diffusion of the released nutrients and supports symplastic nutrient exchange. Fluorescent tracers have shown nutrients moving first into the parenchyma cells, via the symplastic pathway, before being released into the apoplast (Patrick et al., 1995). This passive amino acid efflux is supplemented by amino acid transporters as shown in Pisum sativum and Vicia faba (De Jong et al., 1997; Miranda et al., 2001). In M. truncatula seeds, analysis of transcript and protein distributions highlighted the role of proteolysis in the seed coat as a supplementary source of amino acids for protein synthesis in the embryo (Gallardo et al., 2007). The present transcriptomic analysis in combination with GeneBins showed an over-representation of genes related to all metabolic activity in the seed coat during seed filling at mid maturation (Fig. 3). It is noticeable for the carbohydrate ground parenchyma (gpr); it was subtended by the aleurone layer of embryonic origin (a). (D–F) The structure of vascular bundles is shown: (D) phloem bundles, (E, F) the xylem bundles: (E) in seed section (arrows) and (F) in Nomarski seed micrographs. (G) The chalazal vein (vb) is adjacent to the enlarged parenchyma cells (arrow). (H) Note the branched parenchyma (bpr), with interstitial air spaces adjacent to the vascular bundle (vb). Abbreviations: pe, palisade epidermis; a, aleurone layer; Bar=50 µm in (A), 30 µm in (F), 20 µm in (G), (H), (E), and 15 µm in (B), (C), (D).
metabolism class, with an over-representation of genes annotated as involved in ‘glycolysis/gluconeogenesis’ and starch/sucrose metabolism’ classes (Supplementary Tables S1, S2 at JXB online).

The abundant starch granules transiently accumulated in seed coat cells were detected from the embryo heart stage (8 DAP) until mid maturation (20 DAP). Starch granules are probably a carbon source for embryo growth; their estimated number in the parenchyma correspond to 15 amyloplasts per cell (Fig. 5G–I). The seed coat behaves as a transient storage organ, even if no storage protein transcripts were detected by in situ hybridization (Abirached-Darmency et al., 2005; for a review, see Weber et al., 2005). The overall starch level decreases to 0.6% prior to desiccation, as in lotus and soybean mature seeds (Djemel et al., 2005).

The sucrose status is regulated by seed coat invertases, sucrose synthases, and sucrose transporters, which impact seed composition in P. sativum and V. faba seed (Weber et al., 1996; Dejardin et al., 1997). Invertase (e.g. Mtr.44799.1.S1_at and Mtr.9555.1.S1_at) and sucrose synthase (e.g. Mtr.32293.1.S1_at, Mtr.2239.1.S1_at, and Mtr.22018.1.S1_at) genes are also highly expressed at mid seed maturation in M. truncatula seed coat. More candidate genes of the sucrose status are also shown in Supplementary Fig, S1 and Table S2 at JXB online.

**Late seed coat development and its implication in seed permeability**

The histological analysis of the seed coat in Medicago revealed changes in cell wall thickness of macrosclereids (epidermis), and

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**Fig. 5.** The *Medicago truncatula* seed coat structure during seed filling. (A–C) Light micrographs were stained with the metachromatic toluidine blue. The reddish purple colour of cell walls indicates the presence of large quantities of pectin polysaccharides. (A, B) At early seed filling, 16–20 DAP, in the outer integument, closely packed macrosclereids (ms) are observed under the cuticle (c); they are adjacent to the thick-walled osteosclereids (os) separated by intercellular spaces (arrowhead). (C) Later, at 30 DAP, the seed coat becomes thinner and the parenchyma layers are squashed. (D–I) Transmission electron micrographs of the *M. truncatula* seed coat at 16–20 DAP: (D) The outermost integument layer shows degenerating cells (dc) which form the cuticle(c). (E) The star-like cell (slc) with deep wall invaginations and cytoplasmic erosion is localized beneath the cuticle. (F) Note the vacuole dense accumulations in the macrosclereids (ms), the abundant starch granules (s) in the osteosclereids (os), and the large amount of cell wall thickening. (G) The thin-walled parenchyma cells, adjacent to the endothelium (ed), show many amyloplasts. (H) The endothelial cells were characterized by a compact cytoplasm with no central vacuole. At the interface between the aleurone layer (a) and seed coat, a ‘light line’ (Ll) corresponding to the accumulation of cell wall material was observed. (I) Plasmodesmata are visible in the cell wall, indicating active exchange (arrow). Abbreviations: gpr, ground parenchyma; pr, parenchyma cell; v, vacuoles; e, embryo; sc, seed coat. Bar=20 μm in (A), (B), (C), 4 μm in (D), (E), (F), 2 μm in (G), and 1 μm in (I).
osteosclereids (hypodermis) in the outer integuments throughout seed development (8–20 DAP). A radial elongation of the palisade epidermis was observed, coupled with cell wall thickenings, which finally led to the formation of the closely packed macrosclereid layers. Macrosclereids were 2- to 3-fold their initial size at 20 DAP. They were subtended by irregular osteosclereid cells, which were separated by extensive intercellular spaces. The osteosclereids were distinguished by their most prominent wall thickenings, conferring high rigidity to the seed coat (Fig. 5A, B). The index of cell wall thickenings, using electron microscope image analysis, was estimated to be 20% of the cell volume in parenchyma cells, and 35–45% in macrosclereid and osteosclereid cells. The extreme values (i.e. between 59% and 77%) belong to ‘star-like’ cells localized beneath the cuticle and showing deep invaginations of wall thickenings, which lead to the occlusion of the cell lumen, and cell degeneration (Fig. 5D, E).

Cuticle formation was observed in seed sections at mid-maturation 16–20 DAP, before the end of seed filling, indicating the acquisition of waterproofing in the seed (Fig. 5A, D). The metachromatic toluidine blue stained the pectocellulosic material of the cuticle in pink. The absence of mucilage accumulation was revealed using the ruthenium red stain, which remained negative in M. truncatula mature seeds. However, in Arabidopsis, mucilage secretion produced copious amounts of pectin, which is extruded in the mature seed upon exposure to water (Beeckman et al., 2000; Western et al., 2001; Windsor et al., 2000). Accumulation of cell wall material was also observed at the demarcation line, called the light line, separating the seed coat from the aleuron layer adjacent to the endosperm (Fig. 5H).

A well-documented barrier to prevent water imbibition in the seed coat is suberin accumulation. Suberin is composed of two distinct types of insoluble polyesters of fatty acid and glycerol (reviewed in Nawrath et al., 2002). In Arabidopsis and Medicago, seed permeability was enhanced by mutations affecting suberin biosynthesis which requires the acyltransferase (GPAT5) gene (Liang et al., 2006; Beisson et al., 2007). In the M. truncatula transcriptomic data set, the expression of 12 probe sets containing a glycerol-3-phosphate acyltransferase domain (GPAT) which may be involved in suberin biosynthesis was identified (Supplementary Fig. S3 at JXB online). Out of these 12 probe sets, four GPAT genes were identified as putative orthologues of those reported in soybean (Ranathunge et al., 2010). These genes, expressed in the seed coat, could be targeted to control the biosynthesis of suberin, and to change the degree of permeability, and improve the seed germination rate.

At late seed coat development (16–20 DAP) in M. truncatula, the macrosclereid cells of the outer integument showed abundant accumulation of polyphenolic compounds in their vacuoles (Fig. 5F); their oxidation may also impact seed permeability (Moise et al., 2005; Qutob et al., 2008). Finally, at 30 DAP, when the parenchyma cells were squashed as shown in Fig. 5C, the seed coat became thinner and probably hardened.

In mature seeds, the suberin autofluorescence, previously reported in Arabidopsis (Debolt et al., 2009), was also observed at the hilum in M. truncatula (Supplementary Fig. S6 at JXB online). The external seed coat structure as shown by scanning electron microscopy had a particular papillose surface pattern with caps, except at the hilum region (Fig. 6). These caps probably reinforce the cell wall thickness of the seed coat surface and contribute to its hardness and impermeability in the absence of mucilage production. In contrast to mature seeds from some other legume species (e.g. soybean, pea, and cowpea), the cracks that allowed water absorption were not identified in the seed coat. These observations can explain why seeds require scarification to facilitate water imbibition at germination.

ABA hormonal regulation associated with the seed coat at early seed filling

The signalling networks in plants which are controlled by ABA are extensive, up to 10% of the genes in Arabidopsis. In seeds, the ABA responses associated with embryo maturation are mainly associated with the seed coat (Finkelstein et al., 2002). ABA plays a key role in the regulation of embryo development and seed maturation through its interaction with master regulator genes of seed development ABI3, FUS3, LEC2, and LEC1 (Verdier and Thompson, 2008). In Nicotiana seeds, studies with ABA mutants revealed that ABA is first synthesized in the maternal vegetative tissues and then it is translocated to the seeds to initiate early seed development. The second phase of ABA synthesis, which is needed for embryo maturation, may occur in the seed coat (Frey et al., 2004).

Fig. 6. Scanning electron micrographs of Medicago truncatula mature seed. (A) The mature seed coat showed a papillose surface pattern covered with cell wall caps, one cap per cell, as indicated by arrows. The caps were absent in the hilum region (B). Bar=7 µm in (A) and 5 µm in (B).
Fig. 7. Light microscopy immunolocalization of abscisic acid (ABA) in the *Medicago truncatula* seed coat at the transition stage to seed filling. The micrographs (A, B) correspond to 14 DAP seed sections, and (C, D) to 16 DAP seed sections. In B and D, the ABA monoclonal antibodies were detected as green fluorescence, and were localized in the cell plastids. A and C are the negative controls. The red counterstaining fluorescence was due to propidium iodide. Abbreviations: pe, palisade epidermis; pr, parenchyma; pl, plastids. Bar = 15 µm in (A), and 20 µm in (B), (C), and (D).

The regulation of the ABA biosynthesis pathway in seeds is still to be determined; it is at least known that some of the enzyme substrates may accumulates inside plastids (Nambara and Marion-Poll, 2005). In the present immunolocalization analysis, highly specific monoclonal ABA antibodies conjugated to green fluorescent protein (GFP) were hybridized to the seed sections at early seed filling (Fig. 7). The green fluorescent signals, revealing the presence of ABA, were localized in the cell plastids of the ground parenchyma in the seed coat. These observations suggest that ABA might have a possible endogenous origin in the seed coat, at early seed filling. The endogenous origin was also supported by the transcriptomic analysis, where genes potentially involved in the ABA biosynthetic pathway have a maximum expression in the seed coat at 16 DAP.

The transcript profiling analysis, according to the International Medicago Genome Annotation Group (IMGAG version 3.5), identified 48 probe sets encoding putative proteins of the ABA pathway in the seed coat. They included 22 genes potentially involved in the ABA biosynthetic pathway and 26 genes encoding putative proteins implicated in the transcription of the ABA signal, with ABA-responsive cis-element transcription factors (Supplementary Fig. S4 at JXB online). The hierarchical clustering showed seven genes encoding putative *ZEAXANTHIN EPOXIDASE* proteins clearly expressed in the seed coat, with a maximum expression at 16 DAP (Fig. 8; Supplementary Fig. S5). These genes were involved in the epoxidation of zeaxanthin, which is an early carotenoid precursor of ABA. Five other genes were identified encoding a putative *CAROTENOID CLEAVAGE DIOXYGENASE1* and *9-CIS-EPOXYCAROTENOID DIOXYGENASE 4* proteins, which are responsible for the cleavage of carotenoid precursors to produce xanthoxin, the ABA precursor. This last step of the ABA biosynthesis pathway was considered to be a regulatory step for ABA production according to genetic and biochemical evidence (Zeevaart and Creelman, 1988; Koornneef et al., 1998). Moreover, four *ABSCISIC ALDEHYDE OXIDASE* genes (i.e. two *AAO1* and two *AAO3*) were also expressed in the seed coat, with maximum expression at 16 DAP (i.e. Mtr.42638.1.s1_at and Mtr.5214.1.s1_at; Fig. 8).

According to Medicago gene annotation, all necessary enzymes for ABA biosynthesis are expressed at early seed filling (i.e. 16 DAP), supporting the immunolocalization data for de novo biosynthesis of ABA in the seed coat. The most probable pathway of ABA biosynthesis is in the chloroplasts (Milborrow, 2001; Nambara and Marion-Poll, 2005), which corroborates the ABA immunolocalization in this study. It can be assumed that
ABA adducts allowed ABA immobilization during fixation and resin infiltration, the last steps of ABA biosynthesis being carried out in a form bound to the constitutive compounds of chloroplasts (Netting et al., 1992). This assumption is also supported by the spatio-temporal transcriptomic regulation of the NCED genes which are probably a key element in the control of ABA levels in seeds at early maturation.

Conclusion
This integrated analysis of the seed coat in M. truncatula raised new questions associated with early seed filling. Cell expansion in the seed coat, which is implicated in seed size, could be induced by the onset of the endopolyploidy process. Flow cytometry with microdissection would enable the specific determination of the genome size in the different seed coat tissues (Ochatt, 2008). Putative target genes that may contribute to agronomic seed traits are also provided. The probable participation of the seed coat in ABA biosynthesis at early seed filling and the presence of suberin with its impact on water permeability might be assessed by functional genomic studies using mutant populations in M. truncatula (Tadege et al., 2008; le Signor et al., 2009).

Supplementary data
Supplementary data are available at JXB online.
Figure S1. K-means clustering of the 30 001 genes expressed in seed coat during seed development.
Figure S2. Statistical analysis of fluorescence measurements including the surface of the nuclei (a) and the integrated intensity (b).
Figure S3. The transcriptomic database showed 12 GPAT genes with a phospholipid/glycerol acyltransferase domain which are involved in suberin biosynthesis.
Figure S4. Probe sets encoding genes potentially involved in the ABA biosynthetic pathway and expressed in seed coat.
Figure S5. ABA biosynthetic pathway from Mapman.
Figure S6. Suberin autofluorescence in Medicago truncatula mature seed.
Table S1. List of all probe sets expressed in seed coat.
Table S2. List of probe sets present in each of the four clusters identified using the k-means algorithm during seed coat development.

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