

# ABI5 Is a Regulator of Seed Maturation and Longevity in Legumes

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**The preservation of our genetic resources and production of high-quality seeds depends on their ability to remain viable and vigorous during storage. In a quantitative trait locus analysis on seed longevity in *Medicago truncatula*, we identified the bZIP transcription factor ABSCISIC ACID INSENSITIVE5 (ABI5). Characterization of *Mt-abi5* insertion mutant seeds revealed that both the acquisition of longevity and dormancy were severely impaired. Using transcriptomes of developing *Mt-abi5* seeds, we created a gene coexpression network and revealed ABI5 as a regulator of gene modules with functions related to raffinose family oligosaccharide (RFO) metabolism, late embryogenesis abundant (LEA) proteins, and photosynthesis-associated nuclear genes (PhANGs). Lower RFO contents in *Mt-abi5* seeds were linked to the regulation of *SEED IMBIBITION PROTEIN1*. Proteomic analysis confirmed that a set of LEA polypeptides was reduced in mature *Mt-abi5* seeds, whereas the absence of repression of *PhANG* in mature *Mt-abi5* seeds was accompanied by chlorophyll and carotenoid retention. This resulted in a stress response in *Mt-abi5* seeds, evident from an increase in  $\alpha$ -tocopherol and upregulation of genes related to programmed cell death and protein folding. Characterization of *abi5* mutants in a second legume species, pea (*Pisum sativum*), confirmed a role for ABI5 in the regulation of longevity, seed degreening, and RFO accumulation, identifying ABI5 as a prominent regulator of late seed maturation in legumes.**

## INTRODUCTION

Seed longevity, i.e., the maintenance of viability during storage, is a crucial factor for the conservation of genetic resources and to ensure proper seedling establishment and crop yield. Longevity is progressively acquired during maturation, after seed filling, until the seed reaches a dry, quiescent state (Probert et al., 2007; Verdier et al., 2013; Righetti et al., 2015). During this period of seed development, late embryogenesis abundant (LEA) protein and raffinose family oligosaccharides (RFOs) accumulate, and chlorophyll is degraded (Rosnoblet et al., 2007; Chatelain et al., 2012; Verdier et al., 2013; Righetti et al., 2015). Seed dispersion or harvest in the case of crops can occur any time during late seed maturation (Probert et al., 2007). Therefore, the content of LEA,

RFO, and/or chlorophyll in the seeds is indicative of seed maturity and vigor (Jalink et al., 1998; Sinniah et al., 1998; Vandecasteele et al., 2011; de Souza Vidigal et al., 2016). Also, during seed maturation, the progressive loss of water induces the formation of a so-called cytoplasmic glass, an amorphous matrix resembling a solid-like state where the mobility and relaxation rates of molecules are severely slowed down, thereby imposing a state of quiescence and conferring longevity (reviewed in Buitink and Leprince, 2004).

The role of LEA proteins and small heat shock proteins in longevity has been documented (Hundertmark et al., 2011; Chatelain et al., 2012; Prieto-Dapena et al., 2006). Additional protective factors conferring longevity include a set of antioxidants against oxidation occurring during storage, such as glutathione (Kranner et al., 2006), tocochromanols (Mène-Saffrané et al., 2010; Vom Dorp et al., 2015), and seed coat components, including flavonoids and cutin (Debeaujon et al., 2000; De Giorgi et al., 2015). Seed longevity has also been shown to be associated with DNA and protein repair systems, such as DNA ligase (Waterworth et al., 2010), protein L-isoaspartyl methyltransferase (Ogé et al., 2008), and methionine sulfoxide reductases (Châtelain et al., 2013). The role of RFO in seed longevity remains circumstantial. It is based on observations that RFO contents increase concomitantly with the acquisition of longevity (Sinniah et al., 1998; Verdier et al., 2013). In *Arabidopsis thaliana*, seeds of *alpha-galactosidase2* (*agal2*) mutants exhibited

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reduced longevity (Righetti et al., 2015), and galactinol was found to be a marker for seed longevity in several species (de Souza Vidigal et al., 2016). However, there is no direct evidence for a causal relationship between the protective properties of RFO via a role in the stability of the cytoplasmic glass and longevity (Buitink et al., 2000). Alternative roles for RFOs during establishment of seed longevity include a possible function in carbon storage to provide energy that can easily be mobilized during germination after ageing, a function in mitigating environmental stress and protection against oxidative damage (Nishizawa et al., 2008).

In most plant species, chlorophyll is degraded during the late stages of seed maturation, before the seed reaches the dry state (Nakajima et al., 2012; Delmas et al., 2013; Teixeira et al., 2016). Chlorophyll retention in dry oily seeds appears to be detrimental to seed longevity. Seeds of the *Arabidopsis green-seeded (gr)* mutant contained twice as much chlorophyll as the wild type and exhibited reduced storage stability (Clerkx et al., 2003). Likewise, seeds of the *non-yellow coloring1 (nyc1) nyc1-like (nol)* double mutant affected in the conversion of chlorophyll *b* to chlorophyll *a*, the first step of chlorophyll degradation, contained 10-fold more chlorophyll than the wild type and had a strongly reduced longevity (Nakajima et al., 2012). However, the cause-effect relationship between seed longevity and chlorophyll content remains elusive.

Whereas several upstream regulators of desiccation tolerance have been identified, information is scarce on the regulatory network leading to the acquisition of longevity. *Arabidopsis* loss-of-function mutants of the master regulators *ABSCISIC ACID INSENSITIVE3 (ABI3)* and *LEAFY COTYLEDON1 (LEC1)* produce seeds that lose their viability during desiccation or during the first few weeks after harvest (Ooms et al., 1993; Nambara et al., 1994; Sugliani et al., 2009; Delahaie et al., 2013). Overexpression of the *ABI3*-regulated heat shock transcription factor A-9 from sunflower (*Helianthus annuus*; *HaHSFA9*) led to increased stability during accelerated aging in tobacco (*Nicotiana tabacum*) seeds (Prieto-Dapena et al., 2006; Personat et al., 2014). The A-9 transcription factor also interacts with the sunflower drought-responsive factor *HaDREB2* (DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN B2) in a seed-specific manner to enhance stability during accelerated aging (Almoguera et al., 2009). Using a trait-based gene significance measure and gene network analysis, a coexpression module related to longevity was found that led to the identification of 11 transcription factors, such as *WRKY3* and *NUCLEAR FACTOR, X-BOX LIKE1 (NFXL1)*, whose mutations in *Arabidopsis* led to reduced longevity (Righetti et al., 2015). Another important regulator of seed maturation is *DELAY OF GERMINATION1 (DOG1)*, originally identified for its role in dormancy, but also shown to play a role in seed storage stability and regulation of many seed maturation genes in interaction with *ABI3* (Bentsink et al., 2006; Dekkers et al., 2016).

With the aim of identifying further loci involved in longevity of legume seeds, we performed a quantitative trait loci (QTL) study on a recombinant inbred line (RIL) population of *Medicago truncatula* that we previously used to identify loci for traits related to seed and seedling vigor and nonreducing soluble sugar content (Vandecasteele et al., 2011). Here, we identified *M. truncatula* *ABI5* as a candidate gene involved in longevity through its collocation with a QTL of longevity and further characterized its function during seed maturation in legume seeds. *ABI5* is a member of the

basic leucine zipper (bZIP) family referred to as abscisic acid (ABA) response element binding (AREB) or ABA-responsive promoter element (ABRE) binding factors (ABFs), which mediate cellular responses to ABA in seeds and vegetative tissues (Finkelstein and Lynch, 2000; Jakoby et al., 2002; Fujita et al., 2005; Yoshida et al., 2010; Wang et al., 2015). Acting downstream and/or synergistically with *ABI3*, *ABI5* functions as homo- or heterodimers that bind to ABRE elements found in the regulatory regions of many stress-related genes (Carles et al., 2002; Lopez-Molina et al., 2001; Nakabayashi et al., 2005; De Giorgi et al., 2015) as well as light-signaling genes (Lee et al., 2012; Sakuraba et al., 2014).

In *Arabidopsis*, *ABI5* regulates seed germination, dormancy, and seedling growth. After germination, *ABI5* is necessary for ABA-induced growth arrest under unfavorable conditions (Lopez-Molina et al., 2001) and the reinduction of desiccation tolerance in germinated seeds of *Arabidopsis* (Maia et al., 2014) and *M. truncatula* (Terrasson et al., 2013). *ABI5* also integrates light and ABA signals during germination and seedling growth (Lee et al., 2012; Tang et al., 2013). However, little is known about a possible role of *ABI5* during seed maturation. In *M. truncatula*, *Mt-ABI5* was found to be a hub in the gene network regulating seed survival in the dry state (Verdier et al., 2013), but a role in seed longevity was not investigated.

Using mutants of two legume species, *M. truncatula* and pea (*Pisum sativum*), here, we demonstrate a role for *Mt-ABI5* in the acquisition of seed longevity during seed maturation. Further transcriptomic and biochemical analysis demonstrate a role for *Mt-ABI5* during seed maturation, regulating the accumulation of LEA proteins, RFO, and expression of photosynthetic genes and dismantling of the photosynthetic apparatus. Our results reveal that *Mt-ABI5* is an important regulator of late seed maturation in legumes, connecting degreening with the acquisition of seed longevity.

## RESULTS

### The bZIP Transcription Factor Gene *ABI5* Colocates with QTL for Longevity, RFO Content, and Germination Rate of *M. truncatula* Seeds

To identify *M. truncatula* populations with genetic variability in seed longevity, we screened the parents of different sets of RIL populations. Thus, a strong genotypic difference in longevity was detected between the *M. truncatula* genotypes Jemalong-6 (A17) and DZA315.16, with seed longevity being 4-fold higher in DZA315.16 (Figure 1A). Based on these parental differences, the variability in longevity was assessed in 171 RILs derived from the cross between J6 and DZA315.16 (i.e., the LR-4 population). Two storage conditions were chosen to assess seed life span: (1) 75% relative humidity (RH) at 35°C, known to induce a fast deterioration (Verdier et al., 2013); and (2) 60% RH at 35°C, corresponding to moderate aging. The percentage of final germination and normal seedling morphology was determined after 20 and 159 d of storage under the two conditions, respectively. A large variation was found in these parameters, with germination percentages varying between 10 and 100%, and normal seedling percentages between 0 and 50% (Figure 1B). Ten QTL were detected for seed longevity during accelerated aging, and five during moderate aging, corresponding to six chromosomal regions (Table 1). Three

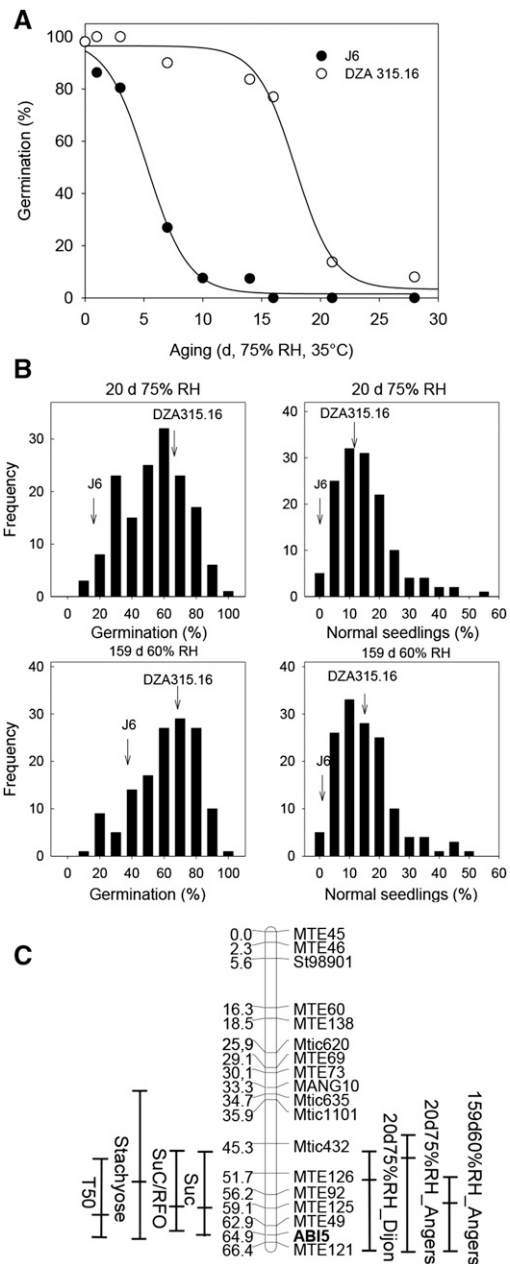
regions showed a colocation between QTL for accelerated aging and moderated aging, explaining between 10 and 21% of the variation (Table 1).

To identify candidate genes that might be responsible for these QTL, genes encoding transcription factors that were previously identified as being correlated with survival in the dry state (Verdier et al., 2013) were localized on the chromosomes. The *Mt-ABI5* gene, a bZIP transcription factor and the homolog of Arabidopsis *ABI5* (Wang et al., 2015), was located at position 64.9 on LG 7, within the confidence interval of three QTL for longevity both for moderate and accelerated aging with seeds obtained from two different growth locations (Angers and Dijon) (Figure 1C, Table 1). This chromosomal region also contained QTL for the composition of soluble sugars Suc and stachyose (Sta), for the ratio Suc/RFO, and a QTL for germination rate, i.e., T50 (time to reach 50% germination; Figure 1C) that were previously identified by our group (Vandecasteele et al., 2011). The *Mt-ABI5* gene was amplified by PCR from both parental lines. Several polymorphisms were detected in the upstream promoter region and intron regions and one single nucleotide polymorphism was found in an exon, resulting in a missense variant R177G (Supplemental Table 1). Mapping of the gene confirmed its localization on linkage group 7 (Supplemental Table 1; Figure 1C).

**Mt-ABI5 Regulates the Acquisition of Seed Longevity and Dormancy during Seed Maturation in *M. truncatula***

The role of *Mt-ABI5* in seed longevity in *M. truncatula* was investigated using *Tnt1* insertion mutants. Previously, we reported ABA insensitivity during germination for two *Mt-abi5* alleles (Terrasson et al., 2013), corresponding to *Mt-abi5-1* and *Mt-abi5-2*. Here, we identified a third insertion mutant allele, *Mt-abi5-3*, for which ABA insensitivity during germination was also confirmed (Supplemental Figure 1). The insertions of the *Tnt1* transposons were all located 5' to the bZIP domain (Figure 2A). To assess the impact of the *Mt-abi5* mutation on seed development, physiological indicators of maturation were monitored from seed filling onwards. No differences in the accumulation of dry weight or the loss of water content during maturation were observed between wild-type and mutant seeds (Figure 2B). Total time of maturation until abscission of the pod was also comparable for all lines, being around 44 d after pollination (DAP). All genotypes acquired their capacity to germinate at the same time at around 15 d. However, in wild-type seeds, time to germination (as assessed by T50) increased during maturation from 10 to 18 d, indicative of the establishment of dormancy (Bolingue et al., 2010; Figure 2C). By contrast, time to germination of *Mt-abi5* seeds steadily decreased during maturation, with a T50 of only 12 h for fully mature seeds (Figure 2C; Supplemental Figure 1). This value was considerably lower than that of completely after-ripened wild-type seeds (T50 = 23 h; Bolingue et al., 2010), indicating that not only dormancy but also germination rate is affected in the *Mt-abi5* mutants.

Seeds of *Mt-abi5* acquired desiccation tolerance at the same developmental stage as wild-type seeds (Figure 2D). Longevity was determined by the construction of survival curves, showing the decrease in germination at the indicated time after storage at 75% RH, 35°C (Figure 2E). Viability of the *Mt-abi5* seeds was lost earlier during storage than that of the wild type. The P50, the time of storage needed to obtain a loss of 50% of germination, was



**Figure 1.** QTL Analysis of Seed Longevity.

**(A)** Loss of viability during storage at 75% RH, 35°C of both parents (J6 and DZA 315.16) using triplicates of 30 to 50 seeds obtained from three to five plants. Data (means ± se) were fitted with sigmoidal curves.

**(B)** Frequency distribution of percentages of germination and normal seedlings after storage obtained from the LR4 RILs. Time and conditions of storage are indicated together with the value obtained from the respective parents.

**(C)** Genetic map of linkage group 7 showing the QTL mapping for time to 50% germination (T50), sugar content, and viability after indicated storage times and conditions from seeds produced in Angers and Dijon (France). The lines indicate the confidence intervals and the peak positions.

**Table 1.** Summary of Detected QTL for Aging Traits in the *M. truncatula* LR4 Population Originating from a Cross between J6 and DZA315.16

Trait	LG	Peak Position (cM)	Confidence Interval (cM)	LOD	R <sup>2</sup> (%)	Additive Effect J6 (%)
20 d aging 75% RH on %FG	1	67.8	62.8–81.5	5.2	13.2	6.9
159 d aging 60% RH on %NS	1	86.3	45–68.3	3.1	9.7	2.8
20 d aging 75% RH on %FG	3	59.3	53.6–62.2	5.4	13.7	–9.4
20 d aging 75% RH on %NS	3	59.3	51.2–62.2	3.7	10.2	–9.4
159 d aging 60% RH on %FG	3	63.5	61.2–66.4	6.2	17.4	–9.8
14 d aging 75% RH on %FG	3	65.5	55.5–66.4	3.6	12.8	–9.1
159 d aging 60% RH on %NS	3	65.5	41.9–66.4	3.5	10.3	–4.1
159 d aging 60% RH on %FG	6	15.1	0–35.6	4.0	12.1	6.6
20 d aging 75% RH on %NS	7	47.3	39.0–66.4	4.0	10.7	–2.1
20 d aging 75% RH on %NS Dijon	7	54.2	47.4–66.6	5.3	13.2	–8.9
159 d aging 60% RH on %NS	7	58.2	52.2–66.4	4.8	14.1	–3.8
20 d aging 75% RH on %NS	8	48.3	40.8–54.6	6.0	15.7	–8.3
14 d aging 75% RH on %FG	8	49.7	43.1–56.1	6.6	21.1	–9.2
14 d aging 75% RH on %NS	8	49.7	41.7–74.7	3.3	11.8	–6.1
20 d aging 75% RH on %FG	8	50.3	41.2–66	5.1	13.0	–6.7

Aging assays were performed at two RHs (60% and 75%) and 35°C. Percentages of final germination (%FG) and of normal seedlings (%NS) were determined at the indicated days of storage. Position in centimorgans (cM) from the LR4 map are described by Vandecasteele et al. (2011). LG, linkage group; LOD, logarithm of the odds; R<sup>2</sup>, percentage of phenotypic variance explained.

consistently reduced in the *Mt-abi5* seeds to 60 to 70% of the wild-type values, for different harvests (Figure 2F).

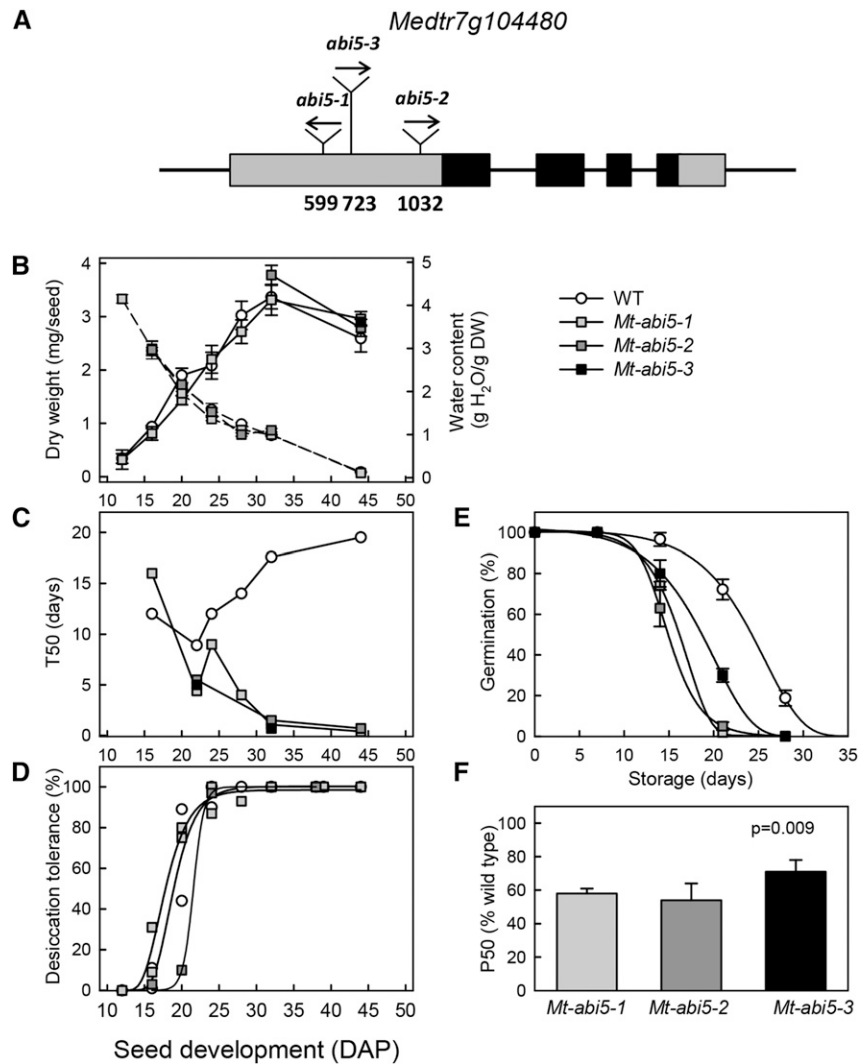
### A Gene Network-Based Analysis of the *Mt-abi5* Transcriptome Reveals Distinct Gene Modules with Specific Biological Functions during Seed Maturation

The reduction of dormancy and longevity in the *Mt-abi5* mutant lines indicates that the late seed maturation is deregulated. To understand the basis for the deregulation, the transcriptomes of wild-type and *Mt-abi5* seeds were analyzed at three stages during seed maturation: 16 DAP (beginning of seed filling); 24 DAP (mid seed filling, when desiccation tolerance is acquired); and pod abscission, when longevity has been fully established. A total of 2042 and 869 probes were up- and downregulated respectively, in *Mt-abi5* mutants compared with wild-type seeds (Supplemental Data Set 1). Venn diagrams showed that this differential expression occurred mostly at abscission, with 1868 (91%) and 730 (84%) transcripts exhibiting increased and decreased levels, respectively (Supplemental Data Set 1). Only a few probes differed between *Mt-abi5* and the wild type at 16 DAP (17 up- and 66 downregulated) and 24 DAP (28 up- and 57 downregulated) (see Venn diagram in Supplemental Data Set 1). Using the seed maturation transcriptome that we previously generated (Righetti et al., 2015; see [http://bar.utoronto.ca/efpmedicago/cgi-bin/efpWeb.cgi?dataSource=medicago\\_seed](http://bar.utoronto.ca/efpmedicago/cgi-bin/efpWeb.cgi?dataSource=medicago_seed)), we noticed that differentially expressed probes in developing *Mt-abi5* seeds can be grouped in different sets of expression profiles compared with the wild type. To capture the temporal regulation of these probes during seed development, a coexpression network was constructed using the intensities (transcript levels) of the wild-type and *Mt-abi5* mutant seeds together with those obtained at 15 time points during wild-type seed maturation (Figure 3).

Three main gene modules were detected that regrouped 1147 nodes (probes) with 2409 edges and that are hereafter referred to as modules I, II, and III (Figure 3A). To understand the topology of

the network, nodes were colored based on their expression profile. Expression of most of the probes was deregulated (repressed or increased) at final maturation (Figures 3A and 3B). Module I contained 51 transcripts that were differentially downregulated at 16 DAP in the *Mt-abi5* seeds compared with wild-type seeds (Figure 3A). This module was significantly enriched with Gene Ontology (GO) terms associated with cell wall organization and pectin esterase activity, with gene products predicted to be localized in the cell wall or apoplast (Table 2). Expression profiles of probes in this module in wild-type seeds showed elevated transcripts during late embryogenesis and early seed filling, after which transcript levels decreased (Figure 3B, top).

In module II, three subclusters were identified and analyzed separately. Cluster IIa contained genes with both up- and downregulated transcripts in the *Mt-abi5* seeds compared with wild-type seeds, in which their level increased during the final stages of seed maturation (Figure 3B). Cluster IIb was characterized by overrepresented biological functions related to “embryonic development ending in seed dormancy,” “defense response,” “response to hormone stimulus and temperature,” as well as “secondary processes” and “reproductive structure development” (Table 2). A zoom on part of this cluster containing probes with downregulated transcripts in the *Mt-abi5* seeds compared with the wild type revealed several transcripts encoding LEA proteins including EM1 and several members of the seed maturation protein family (SMP, also referred to as D-34 family; Figure 3C). This cluster also contained homologs of  $\gamma$ -GLUTAMYL CYSTEINE SYNTHETASE (*GSH1*), which catalyzes the first step of glutathione biosynthesis; *NON-PHOTOCHEMICAL QUENCHING1* (*NPQ1*) involved in the xanthophyll cycle; and *SEED IMBIBITION PROTEIN1* (*SIP1*), a raffinose synthase involved in RFO synthesis. Cluster IIb contained probes with decreased transcript levels in the *Mt-abi5* seeds, whereas they increased from mid seed filling onwards during wild-type seed maturation (Figure 3B). This cluster was overrepresented with GO categories associated with photosynthesis, including nodes



**Figure 2.** Mt-ABI5 Affects Seed Physiological Quality during Seed Development.

**(A)** Gene structure of *Medtr7g104480* showing the *Tnt1* insertions in the three *Mt-abi5* alleles. Boxes and lines indicate exons and introns, respectively. The gray boxes indicate the open reading frame and the black boxes indicate the bZIP domain.

**(B)** Changes in seed dry weight and water content during maturation. Data are the means ( $\pm$ SE) of four replicates of five seeds for immature seeds and 60 seeds for mature seeds.

**(C)** Changes in dormancy evaluated as the time necessary to obtain 50% germination. Data from two independent cultures are shown and were obtained from 50 seeds for each time point.

**(D)** Acquisition of desiccation tolerance evaluated after fast drying and imbibition using 30 to 50 seeds from a pool of 20 plants.

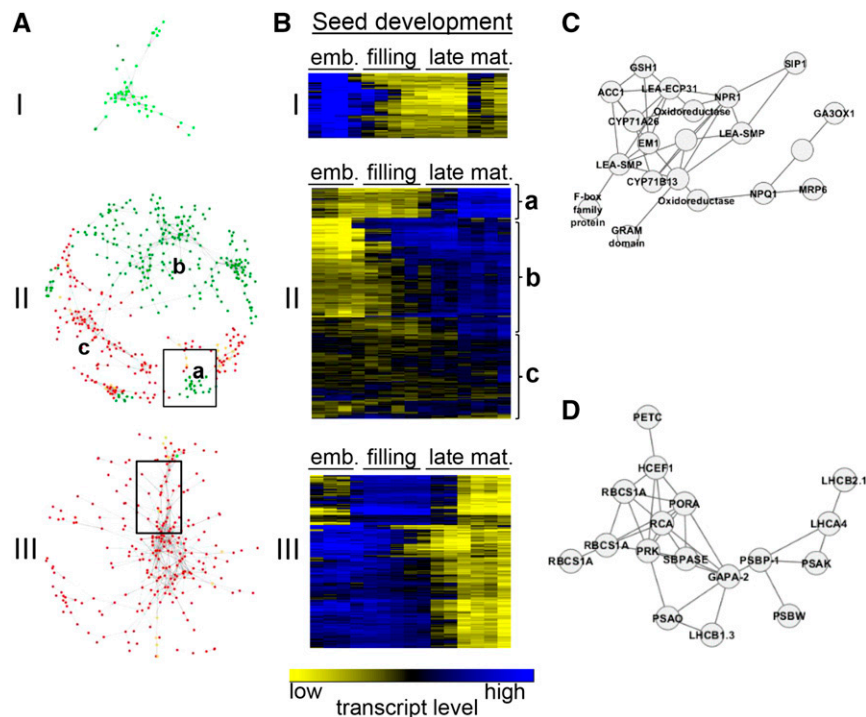
**(E)** Representative loss of viability during storage at 75% RH, 35°C using triplicates of 30 to 50 seeds from a pool of 20 plants. Data (means  $\pm$  SE) were fitted with sigmoidal curves to estimate the P50 (time necessary to obtain a loss of viability of 50% during storage).

**(F)** Longevity of *Mt-abi5* seeds as assessed by calculating P50 during storage of seeds obtained from two to four independent cultures and storage experiments. Data (means  $\pm$  SE) are expressed as percentage of wild-type seeds from plants grown concomitantly with mutants. The level of significance (P) between mutants and the wild type using an ANOVA test (see Supplemental Data Set 3) is indicated.

representing 14 probes encoding chloroplastic Arabidopsis homologs (Table 2). Cluster IIc contained probes with increased transcript levels in the *Mt-abi5* mutants during late maturation compared with wild-type seeds. Most of these probes did not show differential regulation during wild-type seed development and transcript levels were low (Figure 3B). This cluster was over-represented by genes related to programmed cell death and protein

folding, including many small heat shock proteins and chaperonins (Table 2), indicating a stress response in developing mutant seeds.

Module III contained 253 probes with transcripts that were higher in the *Mt-abi5* seeds at pod abscission compared with wild-type seeds (Figure 3A). In wild-type seeds, these genes are highly expressed during embryogenesis and seed filling, while expression decreases during late seed maturation (Figure 3B), indicating that



**Figure 3.** Gene Coexpression Network of Deregulated Transcripts in *Mt-abi5* Mutant Seeds during Development.

**(A)** Gene coexpression modules (I to III) during development of *Mt-abi5* seeds. Temporal analysis of nodes is depicted by coloring genes according to their specific expression in comparison with wild-type seeds. Light-green nodes represent downregulated transcripts in *Mt-abi5* seeds at 16 DAP. Dark-yellow nodes represent upregulated transcripts in *Mt-abi5* seeds at 24 DAP. Dark-green and dark-red nodes correspond to down- and upregulated transcripts, respectively, in *Mt-abi5* at 40 DAP (mature seeds). Gene interactions were visualized using Cytoscape using an edge-weighted spring-embedded layout. **(B)** Expression profiles of the nodes shown in **(A)** during seed development in wild-type seeds. Data are derived from Righetti et al. (2015). The color scale corresponds to transcript level (low = yellow; high = blue). Emb, embryogenesis; late mat., late maturation. **(C)** Focus on the regulation of genes involved in protection against desiccation (box shown in module IIa). **(D)** Focus on the regulation of genes involved in photosynthesis (box shown in module III).

this downregulation is impaired in the *Mt-abi5* mutants. GO enrichment analysis showed a strong overrepresentation in categories associated with photosynthesis and light response (Table 2). Figure 3D shows a detail of the module enriched in these genes: many encode subunits or core units of both PSI and PSII: *PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE1* (*LHCA1*), *PHOTOSYSTEM I SUBUNIT D-2* (*PsaD-2*), *PHOTOSYSTEM I SUBUNIT F* (*PsaF*), *PsaK*, *PsaG*, and *PsaH-2* for PSI and *LIGHT HARVESTING COMPLEX OF PHOTOSYSTEM II 5* (*Lhcb5*), *PHOTOSYSTEM II SUBUNIT P* (*PsbP*), *PsbQ*, and *PHOTOSYSTEM II REACTION CENTER W* (*PsbW*) for PSII. Likewise, a number of genes encoding proteins involved in the Calvin cycle are part of this cluster, as well as a *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A* (*PORA*), involved in the reduction of protochlorophyllide to chlorophyllide (Figure 3D, Table 2).

#### **Mt-ABI5 Regulates Nonreducing Sugar Metabolism and Accumulation of Longevity-Associated LEA Proteins during Late Seed Maturation**

The presence of an *Arabidopsis* homolog of the raffinose synthase *SIP1* in gene module IIb together with the collocation of RFO and longevity QTL suggests a role of *Mt-ABI5* in regulating nonreducing sugar metabolism during seed maturation. To test this hypothesis,

soluble sugars were determined in *Mt-abi5* and wild-type seeds at two time points during seed development: 32 DAP, corresponding to the end of seed filling, beginning of maturation drying, and chlorophyll loss; and maturity, being around 44 DAP. In wild-type seeds, Suc content decreased between 32 DAP and mature seeds, whereas Stac, the major RFO in *M. truncatula* and *verbascose* (*Ver*) contents increased (Figures 4A to 4D). At 32 DAP, *Mt-abi5* seeds contained already significantly more Suc (ANOVA, P value < 0.001; Supplemental Data Set 3), and this difference remained detectable in mature seeds (Figure 4A). By contrast, no significant difference was observed in Stac and *Ver* contents at 32 DAP between *Mt-abi5* and wild-type seeds, whereas a slight difference was detectable for Raf. During further maturation, Stac and *Ver* accumulation also were compromised in the *Mt-abi5* seeds (Figures 4C and 4D). Consequently, the ratio of RFO/Suc was lower for seeds of the three *Mt-abi5* mutant alleles, suggesting that the conversion of Suc into RFO is in part under regulation of *Mt-ABI5* during maturation drying (Figure 4E). A possible role for this conversion could be through the transcriptional regulation of *SIP1* from gene module IIb (Figure 3C). RT-qPCR analysis confirmed that the transcript level of this gene was strongly reduced in mature *Mt-abi5* mutant seeds (Figure 4F). In silico analysis of the upstream promoter sequence of *SIP1* revealed the presence of an ABRE, suggesting a possible binding site for

**Table 2.** GO Enrichment Analysis of Maturation Modules Obtained from Gene Coexpression Analysis

GO Term	Ontology	Description	No. in Input List	#BG/Ref	FDR
Module I					
GO:0042545	P	Cell wall modification	4	386	0.0081
GO:0030599	F	Pectin esterase activity	4	148	1.60E-11
GO:0005618	C	Cell wall	10	618	7.90E-20
GO:0048046	C	Apoplast	5	406	1.70E-06
Module II, cluster a					
GO:0009793	P	Embryonic development ending in seed dormancy	6	465	0.00014
GO:0048316	P	Seed development	6	530	0.00046
GO:0006952	P	Defense response	7	766	0.00082
GO:0009725	P	Response to hormone stimulus	8	982	0.00091
GO:0009628	P	Response to abiotic stimulus	10	1471	0.0012
GO:0009266	P	Response to temperature stimulus	5	485	0.0036
GO:0019748	P	Secondary metabolic process	5	489	0.0036
GO:0048608	P	Reproductive structure development	7	978	0.0076
Module II, cluster b					
GO:0015979	P	Photosynthesis	7	162	5.00E-19
GO:0006091	P	Generation of precursor metabolites and energy	5	285	0.00028
GO:0006952	P	Defense response	7	766	0.023
Module II, cluster c					
GO:0006915	P	Apoptosis	4	159	2.80E-07
GO:0012501	P	Programmed cell death	4	244	0.00035
GO:0006457	P	Protein folding	4	275	0.00083
GO:0009408	P	Response to heat	3	161	0.0011
GO:0006952	P	Defense response	6	766	0.0088
GO:0009743	P	Response to carbohydrate stimulus	3	240	0.036
Module III					
GO:0015979	P	Photosynthesis	13	162	5.30E-26
GO:0010218	P	Response to far red light	5	57	3.70E-09
GO:0009637	P	Response to blue light	5	69	2.40E-07
GO:0006066	P	Alcohol metabolic process	9	270	2.10E-05
GO:0006629	P	Lipid metabolic process	16	841	0.00043
GO:0009416	P	Response to light stimulus	12	596	0.0024
GO:0006461	P	Protein complex assembly	5	134	0.0031
GO:0009314	P	Response to radiation	12	613	0.0031
GO:0009409	P	Response to cold	8	328	0.0047
GO:0009699	P	Phenylpropanoid biosynthetic process	5	141	0.0047
GO:0006952	P	Defense response	12	766	0.04

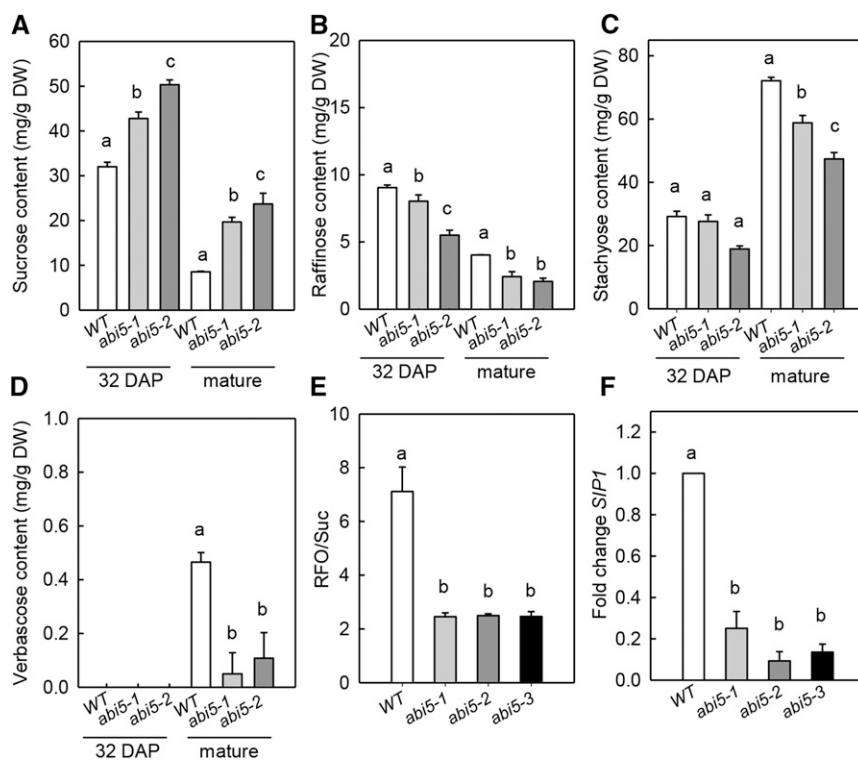
Analysis was performed using AgriGO applying a  $\chi^2$  test with the Yekutieli multitest adjustment method. #BG/ref, number of genes annotated in GO term in background; FDR, false discovery rate; P, biological process; F, molecular function, C, cellular component.

Mt-ABI5. Yeast one-hybrid assays confirmed that a 56-bp promoter fragment of Mt-SIP1 containing the ABRE interacted with the Mt-ABI5 protein, whereas this interaction was abolished when the ABRE was mutated (Supplemental Figure 2).

In addition, module IIa contained several transcripts encoding LEA proteins (Figure 3C). For a number of these genes, we previously reported that their corresponding polypeptide abundance increased in parallel with the acquisition of longevity in *M. truncatula* seeds (Chatelain et al., 2012). Considering the posttranscriptional regulation of these LEA proteins (Verdier et al., 2013), we investigated whether the decrease in LEA transcripts in Mt-*abi5* seeds also resulted in decreased LEA protein accumulation. A protein fraction enriched in LEA polypeptides was isolated, taking advantage of their hydrophilic and unstructured character, which allows them to remain in solution after heating the crude protein extract at 95°C (Boudet et al., 2006). The polypeptides present in this so-called “heat-stable” fraction were previously identified to

establish a reference map for subsequent characterization of the LEA proteome in *M. truncatula* (Chatelain et al., 2012 for A17; Delahaie et al., 2013 for R-108) (Supplemental Figure 3A).

The abundance of seventeen polypeptides was at least twofold reduced in the Mt-*abi5* seeds compared with the wild type (Supplemental Data Set 2 and Supplemental Figures 3B to 3D). The polypeptide showing the strongest change (18-fold) was the EM1 protein, whereas its family member, EM6, was reduced 4-fold (Figure 5A). Both polypeptides together represented 8% of the total spot intensities of the 2D gel (Supplemental Data Set 2). Almost all the polypeptides corresponding to SEED BIO-TINYLATED PROTEIN65 (SBP65) were reduced in abundance (Supplemental Data Set 2). Also, the abundance of all the spots corresponding to D-34 proteins or a mix of D-34 with LeM (spot 100), a mitochondrial LEA protein, was strongly reduced in the Mt-*abi5* seeds (Figure 5B). All of the transcripts of these LEA proteins were downregulated in the Mt-*abi5* mutants, except for



**Figure 4.** Sugar Metabolism Is Affected in *Mt-abi5* Seeds.

(A) to (D) Contents in sucrose, raffinose, stachyose, and verbascose in wild-type and *Mt-abi5* seeds harvested at 32 DAP and at pod abscission (mature). (E) Ratio of RFO/Suc. Data are the average ( $\pm$ se) of four replicates of 10 seeds each from a pool of 20 plants.

(F) Expression of *Mt-SIP1* (*SEED IMBIBITION PROTEIN1/RAFFINOSE SYNTHASE*) in mature wild-type and *Mt-abi5* seeds. Expression was normalized using *Mt-MSC27* and *Mt-ACTIN11* as reference genes. Data are the means of three independent biological replicates. Significantly different values (assessed by ANOVA,  $P < 0.05$ ; Supplemental Data Set 3) were ranked into groups as indicated by the respective letter using the Student-Newman-Keuls test.

LeaM. This suggests that the reduction of intensity of spot 100 is due to a reduction in D-34 rather than LeaM.

Four highly abundant LEA polypeptides, comprising 13% of the total spot intensity, were over 2-fold more abundant in the heat-stable proteome of the *Mt-abi5* seeds compared with the wild type. They corresponded to PM10, a dehydrin (DHN), a member of the D113 family D-113 II (spot 43), and a CapLEA polypeptide (spot 38) (Figure 5C). These results corroborated the transcriptomic analysis in which a significant upregulation was found for D-113 II and CapLEA (Supplemental Data Set 1). Next to LEA polypeptides, three polypeptides that showed the highest increase in abundance in the *Mt-abi5* seeds compared with the wild type were identified as GLYCINE-RICH PROTEIN2, PROTEIN DISULFIDE ISOMERASE and CHLOROPLAST CHAPERONIN10 (CPN10), all of which were involved in protein folding upon the stress response (Supplemental Data Set 2).

#### Chloroplast Pigment Composition and Photosystem-Associated Gene Expression Are Altered in Mature *Mt-abi5* Seeds

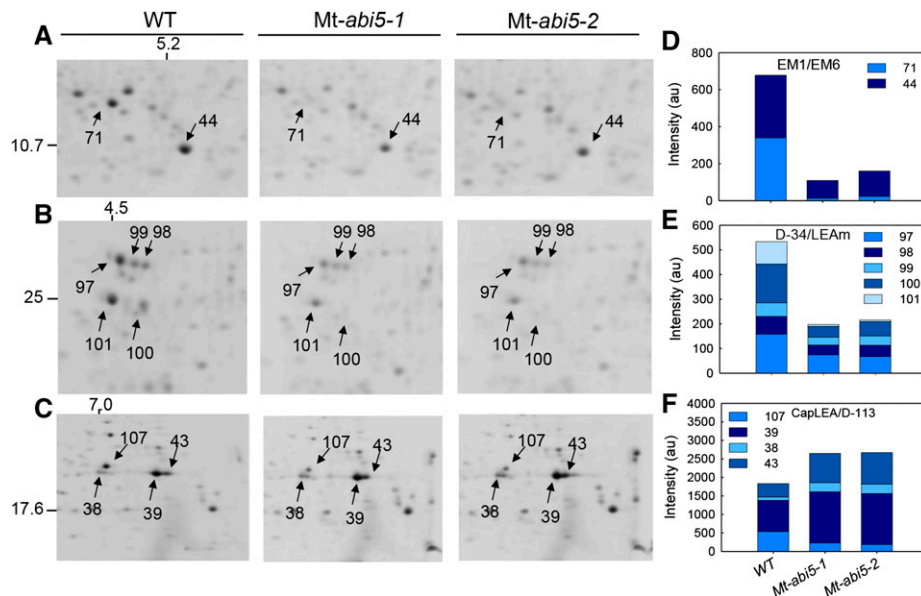
Considering that coexpression modules affected by *Mt-abi5* were enriched with categories associated with photosynthesis genes (Figure 3, Table 2), we investigated whether chlorophyll degradation

was also affected (Figures 6A and 6B). In mature wild-type seeds, only a residual amount of chlorophyll *a* was detected, whereas by contrast, *Mt-abi5* seeds contained 3- to 10-fold more chlorophyll *a* than the wild type (Figure 6A). The *Mt-abi5* seeds also contained small amounts of chlorophyll *b*, whereas it had completely disappeared from wild-type seeds (Figure 6B). During seed development, a difference in chlorophyll degradation was already evident at 24 and 32 DAP, although the differences were not significant due to high variation between the biological replicates (Supplemental Figure 4).

Chlorophyll *b* must be converted to chlorophyll *a* by NYC1 or NOL as a first step toward chlorophyll degradation (Christ and Hörtensteiner, 2014). Therefore, we assessed whether the expression of genes involved in the chlorophyll breakdown pathway was affected in *Mt-abi5* seeds (Supplemental Figure 5). No significant difference in *Mt-NYC1* expression between wild-type and mutant seeds was detected by RT-qPCR (Supplemental Figure 5B). A closer inspection of the transcriptome data showed that the levels of transcripts encoding enzymes involved in the degradation of chlorophyll were either not significantly different or higher in *Mt-abi5* compared with mature wild-type seeds (NYC1, NYE1, and PHEOPHORBIDE A OXYGENASE [PAO]) (Supplemental Figure 5C; Supplemental Data Set 1).

ABI5 has been shown to be involved in the delay of dark-induced senescence in *Arabidopsis* seedlings (Sakuraba et al., 2014). In





**Figure 5.** Mt-ABI5 Affects LEA Polypeptide Accumulation.

(A) to (C) Focus on specific areas of a representative 2D gel showing differentially expressed polypeptides between mature wild-type and Mt-*abi5* seeds. Spot numbers indicate the following polypeptides. (A): 44, EM6; 71, EM1. (B): 97, D34-I; 98, D-34-I/LEAm; 99, LEAm; 100, PM25/D35-II; 101, D34-II/PM25. (C): 39, 38, 107, Cap LEA 1; 43, Protein D-113.II.

(D) to (F) Polypeptide abundance of the indicated LEA polypeptides. Data are the average of spot intensities obtained from five to six gels.

contrast to *Arabidopsis*, no difference could be observed in dark-induced senescence in leaves from the *M. truncatula* Mt-*abi5* mutant compared with the wild type. Whereas chlorophyll content decreased during senescence, no significant difference was detected between the Mt-*abi5* mutant plants and wild-type lines (Supplemental Figure 6). Therefore, none of these findings support a direct role for Mt-ABI5 in the chlorophyll degradation at the final steps of maturation.

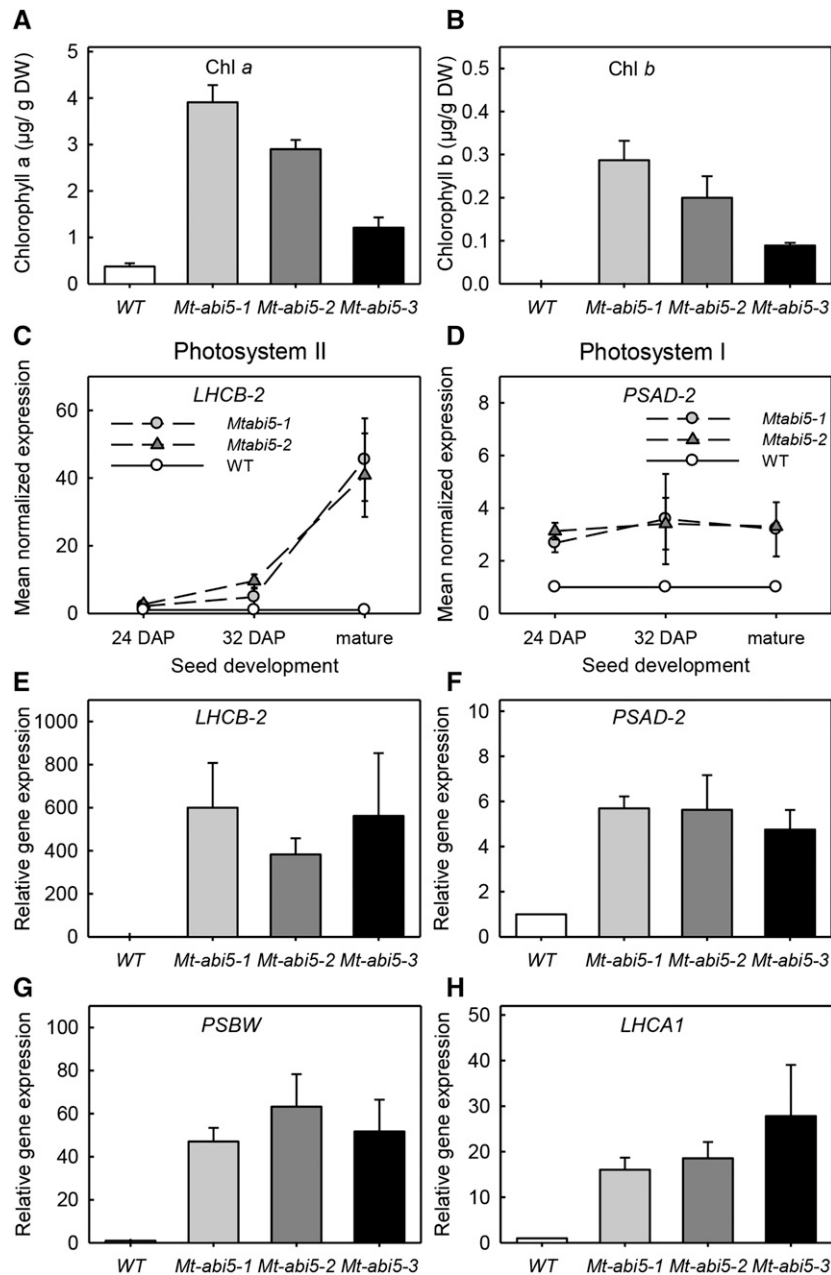
The transcriptome analysis identified numerous transcripts from genes encoding different subunits of PSI and PSII that remained high in the mature Mt-*abi5* seeds, whereas transcripts were completely degraded in wild-type seeds (Figure 3; Supplemental Figure 7A). We confirmed these observations by RT-qPCR analysis on a new harvest of Mt-*abi5* and wild-type seeds (Figures 6C to 6H; Supplemental Figure 7B). Transcript levels of Mt-*LHCB-2* and Mt-*PSAD-2*, belonging to PSII and PSI, respectively, were already 2-fold higher at 24 DAP in Mt-*abi5* seeds compared with the wild type (Figures 6C and 6D). During seed maturation, this difference increased progressively for Mt-*LHCB-2*, resulting in relative amounts of transcripts that were 50-fold higher in mature Mt-*abi5* seeds compared with the wild type (Figure 6C), whereas the difference remained stable for Mt-*PSAD-2* (Figure 6D). Transcript levels for several genes increased 6- to 600-fold in mature Mt-*abi5* seeds (Figures 6E to 6H), with the strongest changes for transcripts of PSII (Figures 6E and 6G).

Carotenoids are present in the reaction center and, like chlorophyll, are known to decrease during seed maturation (Gonzalez-Jorge et al., 2013; Monma et al., 1994). Therefore, to support the hypothesis that chloroplasts are not entirely degraded in Mt-*abi5* mutants, we assessed whether the steady state level of carotenoids

was affected in Mt-*abi5* seeds during maturation by HPLC (Table 3). The decrease in the carotenoids  $\beta$ -carotene and lutein started between 24 and 32 DAP in both wild-type and Mt-*abi5* plants, but no significant differences were found between the genotypes at these stages (Supplemental Figure 7C). However, in mature Mt-*abi5* seeds,  $\beta$ -carotene and lutein contents were 2- and 5- to 6-fold higher, respectively (Table 3; Supplemental Figure 7C). We also detected two other carotenoids that were significantly more abundant in mutant seeds, tentatively identified as zeaxanthin and antheraxanthin, based on their retention time and absorbance spectra of the peaks (Thayer and Björkman, 1990; Table 3).

#### Analysis of *Ps-abi5* Mutant Seeds Confirms a Role of ABI5 in Longevity and Chlorophyll Degradation in Legume Seeds

Longevity is affected in the Mt-*abi5* mutant seeds, whereas, to the best of our knowledge, this phenotype has not been reported for *Arabidopsis abi5* mutant seeds. Therefore, we characterized *abi5-1* seeds of *Arabidopsis* (Finkelstein, 1994). We confirmed the ABA insensitivity and a strong reduction of the LEA EM1 polypeptide in mature seeds of the *Arabidopsis abi5-1* mutant by immunoblotting (Supplemental Figures 8A and 8B). Soluble sugar contents were affected, with Suc levels accumulating to a lesser extent in *abi5-1* mature seeds, whereas total RFO was slightly higher, shifting the RFO ratio from 4.6 in the wild-type to 3.8 in *abi5-1* (Supplemental Figures 8C and 8D). Yet, whereas the Raf content was higher, the amounts of longer oligosaccharides Stach and Ver were lower. These data suggest that the conversion from Raf to Stach and Verb is impaired. However, the other phenotypes related to late maturation that are prominent in Mt-*abi5* mutants were less evident in the



**Figure 6.** Chlorophyll and Photosystem-Associated Gene Expression Are Affected in *Mt-abi5* Seeds.

(A) and (B) Chlorophyll *a* and chlorophyll *b* contents in mature seeds. Data are the means ( $\pm$ SE) of three replicates of 100 seeds from a pool of 20 plants. (C) and (D) Level of expression of *Mt-LHCb-2* and *Mt-PSAD-2* during seed maturation at 24 and 32 DAP and in mature seeds. Data were normalized using expression values found for wild-type seeds at each specific stage. (E) to (H) Level of expression of PSII genes (*Mt-LHCb-2* and *Mt-PSBW*) and PSI genes (*Mt-LHCA1* and *Mt-PSAD-2*) in mature wild-type and *Mt-abi5* seeds. Data were normalized using expression values of wild-type seeds. *Mt-MSC27* and *Mt-ACTIN11* were used as reference genes. Data are the means ( $\pm$ SE) of three independent biological replicates. All values for *Mt-abi5* seeds were significantly different from the wild type, except for *Mt-LHCb-2* gene expression at 24 DAP.

*abi5-1* seeds of *Arabidopsis* (Supplemental Table 2). A slight defect in dormancy was observed for *abi5-1* seeds that were harvested at 19 DAP since 93% of these immature seeds germinated whereas 66% of germination was observed for wild-type seeds (Supplemental Figure 8E). Depending on the growth conditions and

the maturity stage, longevity was not affected or was slightly increased in the *abi5-1* mutant (Supplemental Figure 8E). In addition, no significant difference was found in chlorophyll content, although a tendency for higher chlorophyll contents was observed for mutant compared with wild-type seeds (Supplemental Figure 8F).

Considering these contrasting results in longevity between *M. truncatula* and Arabidopsis, we investigated whether defects in ABI5 would also affect seed maturation and longevity in pea. Pea EMS mutant alleles of *Ps-abi5* were obtained using a TILLING approach (Dalmais et al., 2008), which allowed the identification of three independent nonsense mutations that were located 5' to the bZIP domain (Figure 7A). Mutant plants were backcrossed to the wild-type Cameor line and then cultivated under the same growth conditions before seeds were harvested at maturity. No differences in seed weight or water content were observed between wild-type and mutant seeds. To test their longevity, seeds were stored for increasing periods of time at 45°C and 75% RH and germination was assessed (Figure 7B). *Ps-abi5* seeds exhibited a severe reduction in life span, with a decrease in P50 values of more than 3-fold compared with the wild type (Figure 7B). As in *M. truncatula*, nonreducing sugar contents were also strongly affected in mature *Ps-abi5* seeds, both in the axis (Figure 7C) and cotyledons (Supplemental Table 2). Suc levels increased by 50% in *Ps-abi5* mutant seeds, whereas the major RFO (Stach and Ver) decreased by 1.5- and 2.8-fold, respectively.

*Ps-abi5* and wild-type seeds appeared green when the seed coat was present (Figure 7D). However, a strong difference in the maturation-induced degreening was found after removing the seed coat. Indeed, mutant seeds were much greener than wild-type seeds, particularly in the periphery of the cotyledons (Figure 7D). HPLC analysis of the seed coats revealed no difference in chlorophyll *a* and *b* between the wild type and *Ps-abi5* (Supplemental Figure 9). Biochemical analysis of uncoated seeds showed that chlorophyll *a* and *b* were more abundant in the mutant seeds, both in the periphery and in the center of the cotyledons, with the strongest difference in the periphery (Figure 7E).  $\beta$ -Carotene contents were 2- to 5-fold higher in total seed extracts of mutant seeds compared with the wild type (Figure 7F). Similar differences were found for the tentatively identified carotenoids zeaxanthin and antheraxanthin, whereas lutein content was not affected in *Ps-abi5* seeds. Overall, the phenotype was comparable between *Ps-abi5* and *Mt-abi5* mutants, and even stronger in pea seeds, suggesting that in legumes, ABI5 regulates late seed maturation traits.

### Tocopherol Content Is Affected in Mature *Mt-abi5* Seeds

During chlorophyll breakdown, the phytol released via hydrolysis is the main source of prenyl moieties for tocopherol biosynthesis (Vom Dorp et al., 2015). Seeds accumulate large amounts of tocopherols and other tocochromonols. When the synthesis of these lipophilic antioxidants is impaired during seed development, longevity is strongly affected, due to an increased lipid peroxidation during storage (Mène-Saffrané et al., 2010; Vom Dorp et al., 2015). Therefore, we tested whether the contents of these lipophilic antioxidants were affected in mature seeds of *Mt-abi5* mutants as a result of chlorophyll retention (Table 3).  $\alpha$ -Tocopherol represented more than 90% of the total tocopherol content in seeds of *M. truncatula*. Unexpectedly,  $\alpha$ -tocopherol contents were 20% higher in *Mt-abi5* seeds, whereas other tocochromonols were not significantly affected. Consistent with these observations, *M. truncatula* PHEOPHYTIN PHEOPHORBIDE HYDROLASE (*Mt-PPH*) together with VITAMIN E DEFECTIVE3

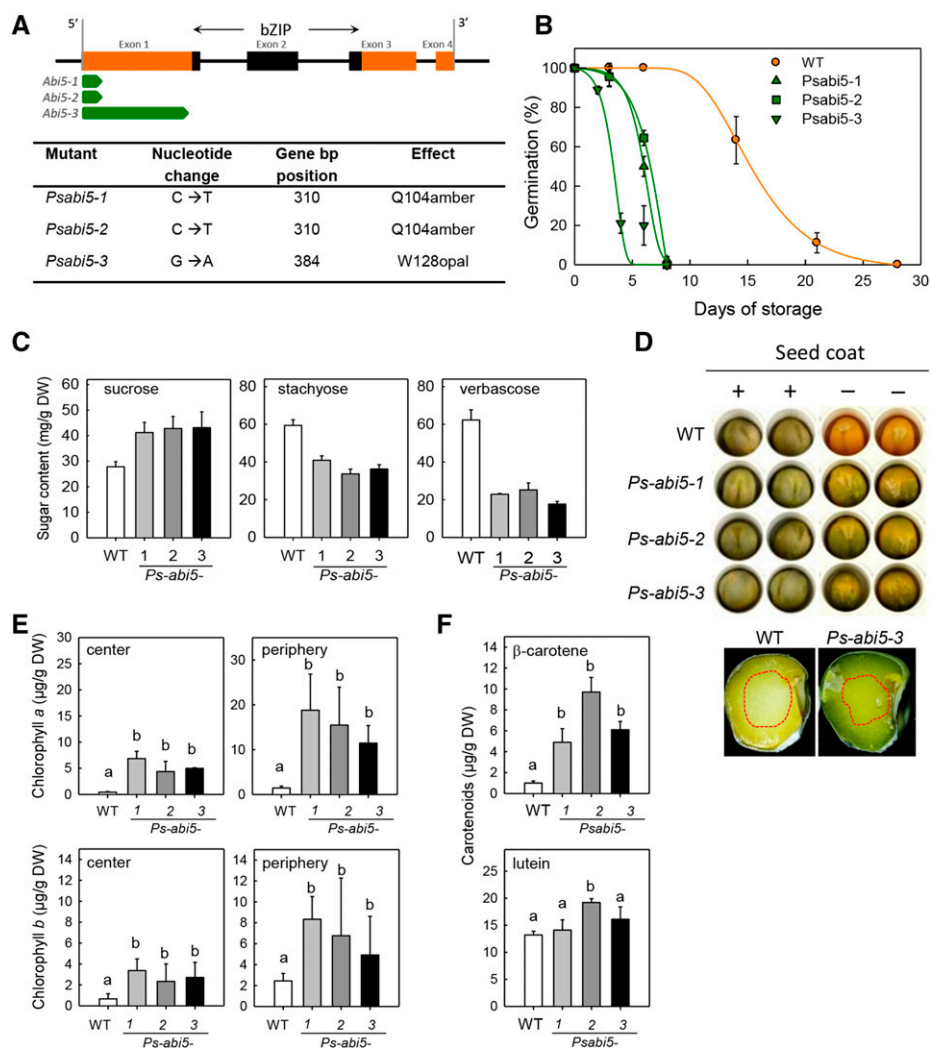
(*Mt-VTE3*) and *Mt-VTE4* transcript levels, involved in chlorophyll hydrolysis, the synthesis of  $\lambda$ -tocopherol, and its conversion to  $\alpha$ -tocopherol, respectively, were higher in mature *Mt-abi5* seeds, but not at 32 DAP (Supplemental Figure 5C and Supplemental Data Set 1). This increase in  $\alpha$ -tocopherol contents might be indicative of an oxidative stress response within the chloroplast in order to protect thylakoid membrane lipids from oxidation (Havaux et al., 2005).

### *Mt-abi5* Seeds Exhibit Enhanced Sensitivity to Oxygen

To further investigate whether *Mt-abi5* seeds are more sensitive to oxidative damage, we performed an aging experiment by storing seeds under  $N_2$  to test whether longevity could be restored in the *Mt-abi5* mutants in the absence of oxygen. Seeds were first equilibrated at 75% RH and then stored hermetically for 14 d at 35°C. When stored in air, *Mt-abi5* seeds showed a significant decrease in viability, whereas the percentage of germination of wild-type seeds was 97% (Figure 8A). When stored under a  $N_2$  atmosphere, the germination percentage of *Mt-abi5* seeds increased sharply and was no longer significantly different compared with wild-type seeds. For those seeds that germinated after storage, we also measured the radicle length after 48 h of imbibition (Figure 8B). Seeds of the three mutant lines did not fully regain their vigor because radicle length remained shorter compared with wild-type seeds, when stored under  $N_2$ , suggesting that additional oxygen-independent mechanisms participated in the deterioration of *Mt-abi5* seeds during storage. We also checked whether high light intensity would impair storage stability and germination of *Mt-abi5* seeds. When stored at 75% RH and 580  $\mu\text{mol photons m}^{-2} \text{s}^{-2}$  for 14 d, the viability of the *Mt-abi5* mutant seeds was not significantly different from that of the wild type. Furthermore, germination in high light intensity did not affect germination, cotyledon greening, or seedling emergence compared with the wild type. Therefore, *Mt-abi5* seeds do not appear to be more susceptible to photooxidation than do wild-type seeds.

## DISCUSSION

Using a QTL approach in *M. truncatula* by screening seeds under accelerated or moderate aging conditions to identify loci of seed longevity, we revealed *Mt-abi5* as a putative regulator of longevity and RFO content. Until now, ABI5 was known to regulate developmental processes during imbibition and seedling establishment, including germination, dormancy, and reestablishment of desiccation tolerance in germinated seedlings (Lopez-Molina et al., 2001; Terrasson et al., 2013; Maia et al., 2014; Supplemental Table 3). Here, the characterization of *M. truncatula* and pea *abi5* mutants revealed that ABI5 governs an array of late maturation-specific events in seeds before reaching the dry state. While seed weight and desiccation tolerance were not affected, both longevity and dormancy, normally acquired during maturation, were severely reduced in *Mt-abi5* and *Ps-abi5* mutants. A network-based approach on seed transcriptomes collected from *Mt-abi5* mutants during maturation revealed a deregulation of several developmentally regulated gene modules with functions related to RFO metabolism, LEA protein accumulation, and regulation of photosynthesis-associated nuclear genes (Figure 9). Mature *abi5*



**Figure 7.** Characterization of *abi5* Mutant Seeds of Pea.

**(A)** Gene structure of *Ps-ABI5* and confirmed *Ps-abi5* EMS mutants detected by TILLING screening. The position of the DNA region encoding the bZIP domain is indicated in black. The open reading frame of the wild-type *ABI5* gene is indicated in orange. The green arrows depict the open reading frames for the mutant lines that carry premature stop codons.

**(B)** Survival curves (percentage of germination) of mature seeds during storage at 45°C, 75% RH. Data are the means ( $\pm$ SE) of three replicates of 30 seeds from a pool of 30 plants.

**(C)** Sugar contents in the axis of mature uncoated pea seeds. Data are the means (SE) of four replicates of 10 seeds from a pool of 30 plants.

**(D)** Representative photographs of dry mature seeds before (+) and after (–) removing the seed coat and after splitting the seed open to show the inside of cotyledons.

**(E)** Chlorophyll contents of mature dry cotyledons. Tissues were harvested in the center and periphery of the cotyledons as outlined in **(D)** (red dashed lines).

**(F)** Carotenoid contents of mature dry seeds. Data from **(E)** and **(F)** are the means of four replicates of 10 seeds from a pool of 30 plants. When significantly different (assessed by ANOVA,  $P < 0.05$ ; Supplemental Data Set 3), values were ranked into groups as indicated by the respective letter using the Student-Newman-Keuls test.

seeds of *M. truncatula* and pea retained chlorophyll and carotenoids (Figures 6A, 6B, 7E, and 7F, Table 3). An additional gene module contained stress response genes whose transcripts were increased during late stages of *Mt-abi5* seed development. The gene modules that show different developmental profiles of expression indicate that *ABI5* is involved in an intricate network with additional transcription factors that regulate pathways pivotal to the seed dispersal characteristics and seed fitness, but

independent from seed filling. It is noteworthy that the total maturation time was not altered between wild-type and mutant seeds. Therefore, *abi5* defects cannot simply be explained by a shorter maturation phase. Transcriptome comparison based on DAP between mutants and the wild type might introduce potential biases due to delayed development. Especially genes that are differential only at one stage based on seed age might be prone to this bias. In our study, only a few probes were differentially

**Table 3.** Contents of Carotenoids and Tocochromanols of Mature Seeds of *M. truncatula*

Compound	Genotype			
	Wild Type	Mt- <i>abi5-1</i>	Mt- <i>abi5-2</i>	Mt- <i>abi5-3</i>
Carotenoids ( $\mu\text{g g DW}^{-1}$ )				
Lutein	1.2 $\pm$ 0.1 (a)	9.6 $\pm$ 0.6 (b)	6.9 $\pm$ 1.2 (b)	7.1 $\pm$ 0.7 (b,c)
$\beta$ -Carotene	1.2 $\pm$ 0.9 (a)	5.0 $\pm$ 0.8 (b)	3.8 $\pm$ 0.3 (c)	2.3 $\pm$ 0.7 (d)
Antheraxanthin	0.2 $\pm$ 0.2 (a)	3.6 $\pm$ 0.2 (b)	2.8 $\pm$ 0.1 (c)	1.8 $\pm$ 0.2 (d)
Zeaxanthin	1.8 $\pm$ 0.3 (a)	10.2 $\pm$ 1.0 (b)	10.0 $\pm$ 0.1 (b)	9.0 $\pm$ 0.6
Tocochromanol (nmol g DW <sup>-1</sup> )				
$\alpha$ -Tocopherol	752 $\pm$ 17 (a)	931 $\pm$ 25 (b)	934 $\pm$ 17 (b)	ND
$\beta$ -Tocopherol	8.8 $\pm$ 1.7	10.5 $\pm$ 0.7	9.5 $\pm$ 0.5	ND
$\lambda$ -Tocopherol	34.0 $\pm$ 0.8	35.3 $\pm$ 2.9	39.9 $\pm$ 2.9	ND
PC-8	3.0 $\pm$ 1.9 (a,b)	0.8 $\pm$ 0.5 (a)	5.8 $\pm$ 1.4(b)	ND

For tocopherols, data are the means ( $\pm$ SE) of five replicates using 20 seeds from a pool of 20 plants. For carotenoids, data are the means ( $\pm$ SE) of three replicates of 100 seeds from a pool of 20 plants. Values for antheraxanthin and zeaxanthins are expressed in  $\beta$ -carotene equivalents. When significantly different (assessed by ANOVA; Supplemental Data Set 3), values were ranked into groups as indicated by the respective letter using a Newman-Keuls test. ND, not determined; PC-8, plastochromanol-8; DW, dry weight.

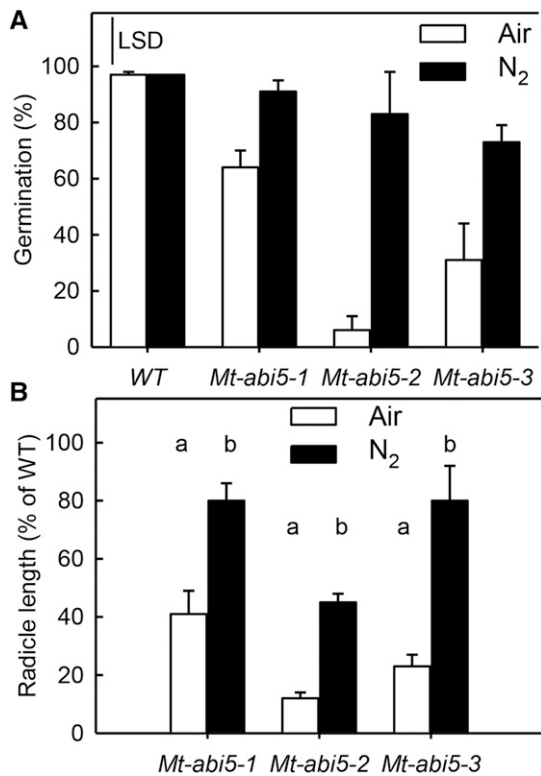
expressed between Mt-*abi5* and the wild type at 16 or 24 DAP (see Venn diagram in Supplemental Data Set 1). For the majority of these probes, differences were found either only at pod abscission or maintained until abscission. In our study, pod abscission corresponds to a physiological stage and is not dependent on the age of the seed, thereby removing this bias.

The chlorophyll retention observed in the mature pea and *M. truncatula abi5* seeds could suggest that chlorophyll degradation is under the regulation of ABI5. In *Arabidopsis* and *Brassica napus* (oilseed rape), chlorophyll retention in mature seeds has been attributed to defects in the first steps of chlorophyll breakdown (Christ and Hörtensteiner 2014), including the conversion of chlorophyll *b* to chlorophyll *a* by NYC1 and NOL (Nakajima et al., 2012) and the oxidation of pheophorbide *a* by PAO (Chung et al. 2006) and STAY-GREEN (SGR), whose function remains to be secured (Christ and Hörtensteiner 2014). In *Arabidopsis* seeds, chlorophyll degradation is under the control of ABI3, NAC016, and ABF4 (Nakajima et al., 2012; Delmas et al., 2013), whereas in senescent leaves, it is under the control of ABI5, since ABI5 binds directly to the promoter of NYC1, NOL, and SGR genes (Sakuraba et al., 2014). However, despite the green phenotypes of pea and *M. truncatula abi5* seeds, our RT-qPCR and transcriptome data showed that genes encoding enzymes of the PAO pathway were either not affected or even upregulated during maturation in the legume *abi5* mutants (Supplemental Figure 5). In addition, chlorophyll breakdown during dark-induced senescence was not affected in Mt-*abi5* seedlings compared with the wild type (Supplemental Figure 6). Thus, chlorophyll retention is likely to be an indirect consequence of a defect in ABI5 that acts on other regulatory mechanisms still awaiting characterization.

Developing Mt-*abi5* seeds also showed a strong deregulation of a large number of photosynthesis-related nuclear and chloroplastic genes (Figure 6; Supplemental Data Set 1). The concomitant regulation of chloroplast and nuclear genes encoding photosynthesis proteins is crucial for the photosynthetic competence of chloroplasts and disassembly during senescence. One of the functions of ABA is to coordinate the expression of these genes in response to developmental and environmental cues (Cutler et al.,

2010; Fujita et al., 2011; Yamburenko et al., 2013). In Mt-*abi5* seeds, this deregulation occurred before the onset of chlorophyll loss since transcript levels of both Mt-*LHCB2* and Mt-*PSAD-2*, encoding subunits of PSII and PSI, respectively, were already 2-fold higher in mutant seeds at 24 DAP (Figures 6C and 6D). During seed maturation, this difference increased progressively for Mt-*LHCB-2*, resulting in relative amounts of transcripts that were 50-fold higher in mature Mt-*abi5* seeds compared with the wild type (Figure 6C). Consistent with this observation, the *Arabidopsis LHCB2* and *FERREDOXIN C2 (FDC2)*; encoding a chloroplastic ferredoxin capable of alternative electron partitioning in PSII) were identified as genes bound by ABI5 *in vivo* in imbibed seeds, suggesting a direct regulation of PSII genes by ABI5 (De Giorgi et al., 2015). Further inspection of the common transcripts upregulated in mature seeds of both Mt-*abi5* and *Arabidopsis abi5-7* (Nakabayashi et al., 2005) revealed 135 differentially expressed genes, out of which five genes were related to PSI, PSII, and light-harvesting complex.

In oily non-chlorophyllous seeds, the presence of chlorophyll in mature seeds is an undesirable trait leading to poor nutritional quality, reduced longevity and economic losses (Jalink et al., 1998; Clercx et al., 2003; Chung et al., 2006; Nakajima et al., 2012; Delmas et al., 2013; Teixeira et al., 2016). Although the link between chlorophyll retention and poor seed quality has been suggested for a long time, a direct cause-effect relationship with longevity has been established only recently. *Arabidopsis* seeds of the *nyc1 nol* double mutant affected in the first step of chlorophyll degradation contained 10-fold more chlorophyll than the wild type and had a strongly reduced longevity (Nakajima et al., 2012). In pea and *M. truncatula abi5* mutant seeds, chlorophyll degradation and longevity were both affected. More chlorophyll was retained in mature dry Ps-*abi5* seeds compared with Mt-*abi5* seeds, and longevity was also much more affected in the Ps-*abi5* seeds (Figures 2E, 2F, and 7B), thus underscoring the detrimental effect of chlorophyll retention in dry seeds. The reasons behind the impaired storage stability observed here but also in seeds of *Arabidopsis* and oilseed rape remain elusive. There are several plausible explanations for this cause-effect relationship that are not mutually exclusive.



**Figure 8.** Mature *Mt-abi5* Seeds Are More Sensitive Than the Wild Type to Oxygen.

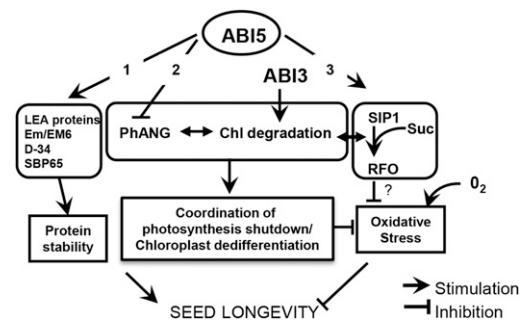
**(A)** Percentage of viability after 14 d of hermetic storage at 35°C under ambient air or nitrogen atmosphere after a 4 d equilibration at 75% RH. Data are the means of triplicates of 50 seeds.

**(B)** Radicle length of aged *Mt-abi5* survivors obtained after 14 d of storage. Data are the means of 10 to 12 radicles ( $\pm$ se) measured after 48 h of imbibition and are expressed as percentage of radicle length of aged wild-type seeds. When significantly different (ANOVA,  $P < 0.05$ ; Supplemental Data Set 3), values were ranked into groups as indicated by the respective letter using a Student-Newman-Keuls test.

The first explanation is that the impaired chlorophyll degradation leads to accumulation of phototoxic products and chlorophyll catabolites. To avoid photooxidative damage and cell death during chlorophyll degradation in leaves and fruits, detoxification of photoactive chlorophyll and chlorophyll catabolites must occur (Christ and Hörtensteiner, 2014). Whether phototoxic products resulting from chlorophyll degradation accumulate in seeds during desiccation is unknown. However, in soybean (*Glycine max*) seeds of the stay-green mutants *cytG* and *Gd<sub>1</sub>d<sub>2</sub>* that retain chlorophyll, the catabolites chlorophyllides and pheophytins were barely detectable (Canfield et al., 1995). In *M. truncatula*, dry, mature *abi5* mutant seeds were not particularly sensitive to high light in comparison to the wild type. Likewise, germination of green seeds from the *grs* and *nyc nol* mutants was not more sensitive to light than darkness (Clerkx et al., 2003; Nakajima et al., 2012). So far, there is no evidence suggesting that phototoxic intermediates accumulate to such an extent that longevity would be impaired.

The second explanation for the link between chlorophyll retention and longevity could be an incomplete regulation of the

chloroplast dedifferentiation that leads to oxidative damage and reduced seed fitness. Several lines of evidence from this work show that developing *Mt-abi5* seeds had to cope with oxidative stress within the chloroplasts during late maturation. To scavenge reactive oxygen species in the chloroplasts, tocopherols and nonphotochemical quenching through the xanthophyll cycle act redundantly, thereby protecting thylakoid membrane lipids from photooxidation (Havaux et al., 2005). In this study,  $\alpha$ -tocopherol contents were increased by 20% in dry mutant seeds and this was accompanied by increased transcript levels of its biosynthetic genes. Furthermore, the operation of the xanthophyll cycle appeared to be disrupted in dry *abi5* mutant seeds from *M. truncatula* and pea: The xanthophyll carotenoids zeaxanthin and antheraxanthin exhibited higher amounts, suggesting an increase in deepoxidation in response to maturation drying (Table 3). The partial restoration of the longevity phenotype when *Mt-abi5* seeds were stored under N<sub>2</sub> also points to a deficiency in the oxidative stress response. When developing *Arabidopsis* seeds were treated with DCMU, which disrupts the electron flow at PSII, mature seeds deteriorated faster during accelerated aging (Allorent et al., 2015). In light of these observations, we speculate that ABI5 might participate in the progressive shutdown of photosynthesis by repressing photosynthesis nuclear gene expression prior to reaching the quiescent dry state. In this way, ABI5 in legumes might be implicated in the coordination of shutting down photosynthesis during seed maturation to avoid oxidative stress before reaching the quiescent state. Our transcriptome and proteome study provides further evidence suggesting that *Mt-abi5* seeds responded to stress during late maturation (Figure 3, cluster IIc). A set of LEA proteins and transcripts of HSP were specifically upregulated, including the chloroplast co-chaperonin 20 (CPN20) as well as other stress-responsive bZIP AREB and ABF transcription factors, such as homologs of AREB3/DPBF2 and AREB1/ABF2, a key regulator enhancing drought tolerance and LEA gene expression (Fujita et al., 2005).



**Figure 9.** Model Summarizing the Different Actions of ABI5 in the Regulation of Late Seed Maturation in Legumes.

(1) Production of LEA proteins Em/Em6, D-34, and SBP65 that protect proteins from aggregation during desiccation/rehydration. (2) Down-regulation of expression of photosynthesis associated nuclear genes, which is accompanied by chlorophyll and carotenoid degradation (although not directly regulated by ABI5) and leads to chloroplast dedifferentiation, which altogether prevents oxidative stress in the mature seed. (3) Induced expression of *SIP1*, thus producing RFO, which might inhibit oxidative stress both inside and outside the chloroplast.

In addition to its role in chloroplast dedifferentiation during maturation drying, ABI5 was found to regulate a set of genes involved in the synthesis of protective compounds (Figures 3 to 5). Previously, it was demonstrated that ABI5 binds directly to the promoters of the *EM1* and *EM6* genes, and Arabidopsis *abi5* mutants exhibited reduced accumulation of the corresponding proteins during maturation (Finkelstein and Lynch, 2000; Carles et al., 2002). Our transcriptome and proteome studies extend these observations as the abundance of several LEA proteins was lower in mutant seeds. In particular, the EM/EM6 proteins, SBP65, and members of the D-34 family were severely reduced. Their role in seed longevity remains to be established, but in vitro, PM25, a member of the D-34 family, and Em6 were found to increase the density of sugar glasses (Tunnacliffe et al., 2010) and act against protein aggregation during rehydration (Boucher et al., 2010). Cytoplasmic glasses severely slow down molecular motion and deleterious reactions, thereby contributing to the stability of the cytoplasm during long-term storage (reviewed in Buitink and Leprince, 2004). Interestingly, the proteome analysis also revealed that several other LEA proteins increased in abundance in the mutants, possibly in an attempt to counteract the absence of the abovementioned LEA proteins. However, this is apparently not sufficient to compensate for the decreased longevity in the Mt-*abi5* mutants.

QTL analysis revealed that three out of five longevity QTL co-located with QTL of RFO composition of seeds from the same harvest (Table 1; Vandecasteele et al., 2011). The discovery that RFO accumulation was affected in the Mt-*abi5* mutants, concomitant with the decrease in longevity, reinforces the genetic link between these two traits. Evidence for a direct link between RFO metabolism and longevity was also recently secured in Arabidopsis by Righetti et al. (2015), who showed that the *agal2* mutant exhibited reduced longevity together with increased Suc and decreased RFO content, as observed in the *abi5* seeds of pea and *M. truncatula*. Consistent with this, the transcript of Mt-*SIP1*, a Raf synthase, was deregulated in the Mt-*abi5* mutant seeds. A direct interaction was demonstrated between Mt-ABI5 and the ABRE in the Mt-*SIP1* promoter (Supplemental Figure 2), suggesting that ABI5 transactivates Mt-*SIP1* expression to regulate the synthesis of RFO. Here, we showed that the Raf to Stach/Ver conversion is also impaired in Arabidopsis *abi5-1* mutants (Supplemental Figure 8), likely via the deregulation of *SIP1*. ChIP-seq analysis identified *SIP1* as a direct target of ABI5 in Arabidopsis (De Giorgi et al., 2015). The role of RFO in seed longevity remains elusive. Our earlier work (Buitink et al., 2000) ruled out a role in the biophysical properties of the cytoplasmic glass state conferring long-term stability to dry seeds. There is evidence that RFO could act as scavenger of reactive oxygen species, in particular protecting photosynthesis from oxidative stress (Nishizawa et al., 2008). Indeed, Raf, being synthesized in the cytosol, can be imported into the chloroplast by an active membrane bound transporter (Schneider and Keller, 2009). In support of this hypothesis, longevity could be partially restored in Mt-*abi5* seeds by storing them under nitrogen. That RFO metabolism might be coupled to chloroplast dedifferentiation and photosynthesis shutdown during maturation is also evident from the observation that DCMU treatment leading to decreased longevity in Arabidopsis (Allorent et al., 2015) also resulted in a 3-fold decrease in galactinol content,

the precursor of RFO. Alternatively, the conversion from Suc to RFO might be related to a sugar signaling pathway as supported by the overrepresentation of Suc-responsive elements in the promoter of genes upregulated during the acquisition of longevity (Righetti et al., 2015).

Whereas in the two legume species, ABI5 played a major role in late maturation processes, related phenotypes in Arabidopsis were less pronounced (Supplemental Table 3). Dormancy, LEA protein accumulation, and RFO metabolism were affected in a similar way, but longevity and chlorophyll retention were not significantly different from the wild-type seeds (Supplemental Table 3). This might be due to redundancy and/or compensation mechanisms among the bZIP family factors involved in ABA signaling in Arabidopsis. ABI5, ABF3, and ABF1 were found to have overlapping effects, and the loss of either factor was compensated for by increased expression of other family members (Finkelstein et al., 2005; Yoshida et al., 2010). In the double mutant *dog1-1 abi3-1*, exhibiting reduced longevity, redundancy was suggested with ABF4 (Dekkers et al., 2016), which also binds to the *NYC1* promoter (Nakajima et al., 2012). The known ability of bZIP transcription factors, such as AREB/ABF for heterodimerization resulting in alternative DNA binding repertoires associated with ABA signaling (Bensmihen et al., 2002; Finkelstein et al., 2005; Yoshida et al., 2010), would therefore provide a combinatorial control of the different seed maturation processes. Such combinations could vary during the course of evolution. In legumes, similar components are present (Wang et al., 2015), but their molecular wiring appears different from Brassicaceae.

To summarize, Figure 9 unveils ABI5 as a master regulator of late seed maturation in legumes by coordinating the acquisition of seed longevity and dormancy via three modes of action: (1) production of LEA proteins Em/Em6, D-34, and SBP65 that protect proteins from aggregation during desiccation/rehydration; (2) downregulation of expression of photosynthesis-associated nuclear genes, which is accompanied by chlorophyll and carotenoid degradation (although probably not directly regulated by ABI5) and leads to chloroplast dedifferentiation, which altogether prevents oxidative stress in the mature seed; and (3) induced expression of *SIP1*, thus producing RFO, which might inhibit oxidative stress both inside and outside the chloroplast. Thus, ABI5 appears to be a prominent regulator of late maturation in legumes, and this finding sheds light on the relationship between seed vigor and RFO accumulation (Vandecasteele et al., 2011) and chlorophyll retention (Jalink et al., 1998; Nakajima et al., 2012; Delmas et al., 2013; Teixeira et al., 2016). Together with previous studies showing a correlation between longevity and chlorophyll retention in Arabidopsis (Clerkx et al., 2003; Nakajima et al., 2012), our data suggest that degreening cannot be uncoupled from long-term survival in the dry state, an important aspect of seed vigor and seedling establishment. This crosstalk offers new opportunities to tackle the green seed problem as well as antinutritional characteristics of RFO in legumes.

## METHODS

### Plant Material

*Medicago truncatula* ssp *tricycla* (R-108) plants were cultivated according to Chatelain et al. (2012). Seeds were harvested upon pod abscission or at

the indicated stages of development and stored at  $-196^{\circ}\text{C}$  in liquid nitrogen. Three independent *Tnt1* insertion lines in the *Mt-ABI5* gene were used: NF4383, referred to as *Mt-abi5-1*; NF3376, referred to as *Mt-abi5-2*, both previously characterized by Terrasson et al. (2013); and NF7680, referred to as *Mt-abi5-3*, that was characterized in this study. *Tnt1* insertions were verified by PCR using forward and reverse primers listed in Supplemental Table 4. The *Mt-abi5-1* line was backcrossed twice with R-108. Pea (*Pisum sativum*) mutants and wild-type (var *Cameor*) were isolated by TILLING according to Dalmais et al. (2008) and grown under greenhouse conditions at  $18^{\circ}\text{C}/22^{\circ}\text{C}$  and a 16-h photoperiod ( $200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) in a mixture of vermiculite and soil. Mutants were backcrossed twice and mutations were verified by dCAPS markers (Supplemental Table 4). Seeds were harvested at shedding.

*Arabidopsis thaliana* ecotype Wassilewskija and *abi5-1* mutants (N8105) (Finkelstein, 1994) were obtained from the NASC. The *abi5-1* mutation was confirmed by DNA sequencing using primers indicated in Supplemental Table 4). *Arabidopsis* seeds were multiplied by growing plants in a growth chamber ( $23^{\circ}\text{C}$ , 16 h light,  $100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ). Seeds were harvested at 19 DAP (*Arabidopsis*) or at shedding. Seeds from *Arabidopsis* and *M. truncatula* were dried after harvest for 2 d at 44% RH,  $20^{\circ}\text{C}$ , and pea seeds were dried at  $20^{\circ}\text{C}$  in air (67% RH) for 2 weeks.

### Phenotyping and QTL Analysis

The LR4 population consisted of 171 RILs and was derived from a cross between A17 and DZA315.16 (Vandecasteele et al., 2011). Longevity was determined on seed samples pooled from three plants. Before measurements, seeds were stored at  $20^{\circ}\text{C}$  at 60% RH for 5 months to allow for dormancy release, after which they were stored for an additional 7 months at  $10^{\circ}\text{C}$  prior to the longevity experiments. Four repetitions of 40 seeds were scarified, equilibrated for 7 d at 60% RH (moderate aging) or at 75% RH (accelerated aging), sealed in aluminum bags (Barrierfoil; Moore and Buckle) and stored for 159 d and 20 d, respectively, at  $35^{\circ}\text{C}$ . After storage, seeds were imbibed on filter paper and the percentages of final germination (radicle protrusion) and of morphologically normal seedlings were scored after 10 d at  $20^{\circ}\text{C}$  in the dark. Petri dishes were randomized following a complete-block design with four blocks, each containing one biological replicate. All statistical analyses were performed with SAS 8.1 (SAS Institute, 2000) as described by Vandecasteele et al. (2011).

Genetic maps and molecular data for the LR4 population are described by Vandecasteele et al. (2011). QTL mapping was performed using the MCQTL software (Jourjon et al., 2005) as described by Vandecasteele et al. (2011). For each QTL identified, additive genetic effect, percentage of phenotypic variance explained ( $R^2$ ), *F*-test value, as well as the confidence interval were determined and equivalent LOD scores calculated (Table 1). The map of linkage group 7 was drawn using MapChart software (Voorrips, 2002). Primers used for the mapping of *Mt-ABI5* are indicated in Supplemental Table 4.

### Physiological Characterization of *abi5* Mutants

For germination assays, three replicates of 30 to 50 scarified seeds of *M. truncatula* or 100 to 200 *Arabidopsis* seeds were imbibed on filter paper with 4 and 1 mL, respectively, of distilled water at  $20^{\circ}\text{C}$  in the dark. For pea, three replicates of 30 seeds were imbibed in 100 mL of distilled water with folded filter paper. Seeds were considered as germinated when the radicle protruded from the seed coat. For ABA sensitivity assays, three replicates of 30 to 50 scarified *M. truncatula* mature seeds were imbibed in the presence or absence of  $10\ \mu\text{M}$  of ABA (Sigma-Aldrich). For *Arabidopsis*, 100 to 200 mature seeds were imbibed on two filter papers in the presence or absence of  $1\ \mu\text{M}$  ABA and incubated for 3 d at  $4^{\circ}\text{C}$  in dark followed by 8 d at  $20^{\circ}\text{C}$  with a 16-h photoperiod.

To assess longevity, mature seeds (scarified for *M. truncatula*) were stored in the dark or in the light ( $580\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) at 75% RH using

hermetically closed containers containing a saturated NaCl solution. Storage temperature was  $35^{\circ}\text{C}$  for *M. truncatula* and *Arabidopsis* and  $45^{\circ}\text{C}$  for pea. At different storage time intervals, seeds were imbibed and final germination percentage was counted after 10 d and 8 d, respectively. For the storage experiment in  $\text{N}_2$ , replicates of 30 seeds were first equilibrated at 75% RH,  $20^{\circ}\text{C}$  for 4 d, then transferred into an aluminum bag and sealed. Bags were pierced with a needle and  $\text{N}_2$  was flushed for 20 s, after which the bag was resealed and placed at  $35^{\circ}\text{C}$ . Controls were seeds that received the same preequilibration treatment and were subsequently stored at 75% RH and  $35^{\circ}\text{C}$  for 14 d. Thereafter, germination was counted and radicle measured after 48 h of imbibition.

For the seed coat permeability assay, four replicates of 5 to 10 scarified mature seeds were incubated at  $20^{\circ}\text{C}$  and 100% RH and weighed from 0 to 8 h. Rates of imbibition were calculated by averaging the rate of water uptake during step increments of 2 h. The leaf senescence experiment was adapted from Sakuraba et al. (2014). Leaves of 4-week-old *M. truncatula* plants were detached and incubated in water at  $21^{\circ}\text{C}$  in the dark. After 0, 4, or 8 d of incubation, photographs were taken and leaves were frozen in liquid nitrogen. Between 2 and 10 mg of lyophilized powder was weighed and chlorophyll was extracted and spectrophotometrically measured as described below.

### Tocopherol Measurement

Five replicates of 20 mature seeds were frozen in liquid nitrogen and ground to a fine powder (Precellys homogenizer). Tocopherols were isolated from the ground tissue using diethylether and  $0.2\ \text{M H}_3\text{PO}_4/1\ \text{M KCl}$ . The upper organic phase was dried under air flow and the lipids were dissolved in *n*-hexane for HPLC analysis (Zbierzak et al., 2009).

### Chlorophyll and Carotenoid Assays

For spectrometry analysis, replicates of 25 *M. truncatula* seeds or 5 to 7 mg of *Arabidopsis* seeds, or leaf material, were lyophilized overnight, weighed, and ground in  $300\ \mu\text{L}$  or  $600\ \mu\text{L}$  of *N,N*-dimethylformamide (Sigma-Aldrich), respectively. After overnight incubation at  $4^{\circ}\text{C}$ , samples were vortexed for 30 s and centrifuged for 5 min at  $10,000g$ . Chlorophyll concentrations were measured according to Moran (1982) on a Fluostar Omega spectrophotometer (BMG Labtech). For HPLC analysis, pigment extraction and quantification were performed as described by Perrin et al. (2016). Briefly, the carotenoid measurements were performed with a Shimadzu HPLC equipped with a thermostated autosampler (SIL-10ADVP) and a diode array detector (SPD-M10AVP). Carotenoids were identified using standards of lutein and  $\beta$ -carotene.

### Sugar Determination

Soluble sugar contents were assessed by HPLC (Dionex) according to Rosnoblet et al. (2007). Analysis was performed on four replicates of 10 mature seeds of *M. truncatula*, four replicates of five mature pea seeds (cotyledons or embryonic axes), and 15 mg of mature *Arabidopsis* seeds.

### RNA Extraction, Microarray Analysis, and Gene Coexpression Network

RNA extraction was performed on three replicates of 50 seeds at 16 and 24 DAP and at abscission, for genotypes R-108, *Mt-abi5-1*, and *Mt-abi5-2*. Seeds were ground in liquid nitrogen. The extraction was performed with the Nucleospin Extract II, Total RNA Purification Kit (Macherey Nagel). RNA amplification, labeling, and hybridization of Nimblegen Medtr\_v1.0 12x135K arrays were performed according to Terrasson et al. (2013). Three biological replicates were analyzed per comparison using the dye-switch method, and statistical analysis on the gene expression



data was performed according to Verdier et al. (2013). Genes were considered differentially expressed when the  $\log_2$  ratio between *Mt-abi5* and the wild type was  $>1$  or  $\leq 1$  at a given time point for each of the two mutants, with a P value  $< 0.01$ . Nimblegen microarray data were deposited in the NCBI Gene Expression Omnibus database (accession no. GSE87313).

The gene coexpression network of the 2911 differentially expressed genes (Supplemental Data Set 1) of the *Mt-abi5* mutants was constructed using the normalized intensities of the wild-type and *Mt-abi5* mutants together with those obtained at 15 time points during seed maturation, derived from Righetti et al. (2015). The network was constructed using Biolayout software (Goldovsky et al., 2005) with a correction value of 0.92, giving rise to a network with 1147 nodes and 2409 edges. Gene interactions were visualized using the open source software Cytoscape (version 3.3.0) with an edge-weighted spring-embedded layout.

### RT-qPCR Analysis

For RT-qPCR, 1  $\mu\text{g}$  total RNA was reverse-transcribed using the Quantitect reverse transcription kit (Qiagen), and qPCR was performed on a CFX96 real-time detection system (Bio-Rad Laboratories) according to the manufacturer's instructions with SsoFast Eva Green Supermix (Bio-Rad Laboratories). Reference genes were *MSC27* (Bolingue et al., 2010) and *Mt-ACTIN11*. Forward and reverse primers used for the different genes in this study are listed in Supplemental Table 4. Relative expression levels were calculated using the comparative  $2\Delta(\text{Ct})$  method (Schmittgen and Livak, 2008). Each data point represents the mean of three biological replicates of 30 seeds.

### Yeast One-Hybrid Assay

Yeast one-hybrid assays were performed using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech) according to the manufacturer's instructions. Primers used for the construction of the prey and bait are indicated in Supplemental Table 4. Fifty-six base pair oligonucleotides of the promoter region of *Mt-SIP1* (−1348 to 1298) containing the ABRE consensus sequence or a mutated version were cloned into the pAbAi vector, carrying the AUR1-C gene. The plasmid was linearized and integrated into the genome of yeast (Y1H Gold). Yeast cells were then transformed with pGADT7-AD plasmid containing the coding region of *Mt-ABI5*. DNA-protein interaction was determined based on the ability of the cotransformants to grow on SD/-Leu medium in the presence of 200 to 500 ng  $\text{mL}^{-1}$  of aureobasidin A.

### Protein Extraction, 2D-PAGE, and Immunoblotting

Soluble protein extraction, preparation of heat-soluble fractions, and analysis using 2D-PAGE were performed according to Chatelain et al. (2012). The experiments were set up in a randomized block design where six gels corresponding to three independent protein extractions of 50 seeds were run in parallel. Polypeptides were identified using the reference gel previously obtained by Chatelain et al. (2012) and Delahaie et al. (2013). Immunoblotting analysis was performed according to Boudet et al. (2006). Briefly, 10  $\mu\text{g}$  of heat-stable protein extract was separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). Immunodetection was performed by chemiluminescence using a rabbit polyclonal antibody raised against the recombinant Mt-EM6 protein (dilution 1:10,000; Davids Biotechnologie), an anti-rabbit IgG-peroxidase conjugate (dilution 1:50,000; Sigma-Aldrich A0545 Lot 32K4882), and the Amersham ECL Plus Western Blotting Detection reagents (GE Healthcare Life Sciences), following the manufacturer's instructions. Chemiluminescence was monitored by a Chemidoc TM XRS molecular imager (Bio-Rad Laboratories).

### Accession Numbers

Sequence data in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: *Mt-ABI5*, Medtr7g104480; *Mt-ACTIN11*, Medtr2g008050; *Mt-MSC27*, TC85211; *Mt-SIP1*, Medtr3g077280; and *Ps-ABI5*, PsCam017634. Transcriptomic data obtained in this study have been deposited in the Gene Expression Omnibus database under accession number GSE87313.

### Supplemental Data

**Supplemental Figure 1.** Germination of *Mt-abi5* seeds is insensitive to ABA during imbibition in the dark.

**Supplemental Figure 2.** Yeast one-hybrid binding assays of *Mt-ABI5* to the ABRE in the promoter region of *Mt-SIP1*.

**Supplemental Figure 3.** Two-dimensional electrophoresis gels of the heat stable proteome of *M. truncatula* seeds.

**Supplemental Figure 4.** Chlorophyll contents in developing wild-type and *Mt-abi5* seeds.

**Supplemental Figure 5.** Expression of genes involved in the PAO degradation pathway in mature seeds of *Mt-abi5*.

**Supplemental Figure 6.** Dark-induced senescence in young seedlings of the wild type and *Mt-abi5*.

**Supplemental Figure 7.** Photosynthesis-associated gene expression and carotenoid content during seed development of wild-type and *Mt-abi5* seeds.

**Supplemental Figure 8.** Longevity of wild-type and *abi5-1* seeds of *Arabidopsis* together with sugar content and EM protein levels in dry mature seeds.

**Supplemental Figure 9.** Chlorophyll contents in seed coats of mature wild-type and *Psabi5* mature seeds.

**Supplemental Table 1.** Detected polymorphisms between Jemalong J-6 and DZA315.16 in the region of *Mt-ABI5* (Medtr7g104480) on chromosome 7.

**Supplemental Table 2.** Contents in soluble sugars of mature pea cotyledons.

**Supplemental Table 3.** Comparative analysis of affected phenotypes in seeds of the *abi5* mutants of *Medicago truncatula*, *Pisum sativum*, and *Arabidopsis thaliana*.

**Supplemental Table 4.** List of primer combinations used for mutant selection and gene expression validation by qRT-PCR.

**Supplemental Data Set 1.** Differentially expressed genes between *Mt-abi5-1* and *Mt-abi5-2* and wild-type (R-108) seeds during maturation.

**Supplemental Data Set 2.** Identification and quantification of polypeptide abundance in mature seeds of *Mt-abi5-1*, *Mt-abi5-2*, and the wild type (genotype R-108).

**Supplemental Data Set 3.** Statistical test results showing the level of significance of data shown in the figures.

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#### AUTHOR CONTRIBUTIONS

O.L. and J.B. conceived the project and research plans, supervised the experiments, and wrote the article with contributions from all authors. J.Z., D.L., E.T., and E.C. performed most of the experiments and analyzed the data with contributions from K. Gutbrod, P.D., C.V., E.G., and K. Gallardo. B.L.V., M.D., and C.D.-L. provided technical assistance. C.L.S., K. Gallardo, M.D., and A.B. performed the genetic screenings of mutant pea and provided the *Ps-ABI5* mutant lines.

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