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Running title: MtABI4 affects seed degreening and longevity

Key words: *Medicago truncatula*, longevity, ABI4, ABI5, degreening, maturation, eoplast, chlorophyll, photosynthesis, stay-green

SUMMARY

Seed longevity, the capacity to remain alive during dry storage, is pivotal to germination performance and essential to preserve genetic diversity. It is acquired during late maturation concomitantly with the seed degreening and de-differentiation of chloroplasts into colorless, non-photosynthetic plastids called eoplasts. Since chlorophyll retention leads to poor seed performance upon sowing, these processes are important for seed vigor. However, how they are regulated and connected to the acquisition of seed longevity remains poorly understood. Here, we show that such a role is provided by ABSCISIC ACID INSENSITIVE 4 in the legume *Medicago truncatula*. Mature seeds of *Mtabi4* mutants contained more chlorophyll than wild-type seeds and exhibited a 75% reduction in longevity and reduced dormancy. MtABI4 was necessary to stimulate eoplast formation as evidenced by the significant delay in the dismantlement of photosystem II during maturation of mutant seeds. *Mtabi4* seeds also exhibited transcriptional deregulation of genes associated with retrograde signaling and transcriptional control of plastid encoded genes. Longevity was restored when *Mtabi4* seeds developed in darkness, suggesting that shutdown of photosynthesis during maturation rather than chlorophyll degradation *per se* is a requisite for the acquisition of longevity. Indeed, shelf life of *stay green* mutant seeds that retained chlorophyll was not affected. Thus, ABI4 plays a role in coordinating the dismantlement of chloroplasts during seed development to avoid damage and compromise seed longevity acquisition. Analysis of *Mtabi4 Mtabi5* double mutants showed synergistic effects on chlorophyll retention and longevity, suggesting that they act *via* parallel pathways.

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Significance statement

Seed degreening occurs at final maturation, and is a process that is crucial for seed performance and longevity. Here, we demonstrate the importance of ABI4 and ABI5 in coordinating the dismantlement of chloroplasts during development to avoid damage, which otherwise compromises seed longevity during maturation drying.

INTRODUCTION

Seed maturation is the period of seed development starting after embryogenesis and terminating with a period of desiccation that brings the seed water content to an equilibrium with ambient air. It is characterized by the accumulation of storage reserves to sustain growth during the heterotrophic phase of seedling establishment and the acquisition of several traits that are pivotal to the performance of the seed dispersion after abscission (Leprince *et al.*, 2017; Finch-Savage and Bassel, 2015). Depending on the species, these traits include desiccation tolerance, dormancy and longevity. Desiccation tolerance is the capacity to tolerate a water loss below 8-10%, whereas longevity is defined as the capacity to remain alive for long periods during storage. Both traits are not only crucial to ensure the regeneration of plants over time when seeds are buried in the soil but also essential to preserve genetic diversity *ex situ* in gene banks. In the industry, poor seed longevity leads to a decrease in the vigor of seed lots, thereby generating unexpected commercial losses.

Longevity is brought by the capacity of the seeds to tolerate a latent quiescent state by drying and cooling, such that integrated metabolism ceases. To confer longevity, a program is activated during seed maturation leading to the synthesis of protective compounds such as late embryogenesis abundant (LEA) proteins, non-reducing sugars and heat shock proteins (HSP) (Chatelain et al., 2012; Righetti et al., 2015; Sano et al., 2016). The capacity to avoid oxidation during storage is also an important factor contributing to longevity (Roach *et al.*, 2018; Sano *et al.*, 2016; Renard *et al.*, 2020; De Giorgi *et al.*, 2015). In the dry state, the cellular content is transformed into a glassy state corresponding to a solid-like stabilized matrix in which molecular motion is severely slowed down, thereby drastically reducing degradative reactions and enzyme reactions (Buitink *et al.*, 2000; Ballesteros *et al.*, 2019; Fernández-Marín *et al.*, 2013; Ballesteros and Walters, 2019).

Seed maturation is also characterized by dramatic changes in the photosynthetic activity. During seed filling, chloroplasts are well adapted to low light conditions and fully active. Their function is crucial to ensure seed germination and seed vigor (Kim *et al.*, 2009; Allorent *et al.*, 2015; Sela *et al.*, 2020) At the end of seed maturation, chloroplasts de-differentiate into colourless, non-photosynthetic plastids called eoplasts by dismantling light harvesting complexes (LHC) of photosystem II (PSII) and photosystem I (PSI) and degrading Chl *a* and *b* via pathways that are poorly understood (Kim et al., 2009b; Smolikova et al., 2018). No photosynthetic activity is detected in dry seeds (Smolikova *et al.*, 2018; Vertucci *et al.*, 1985).. In senescing leaves, chloroplast dismantling has been described as a complex process that requires the coordination of intra- and extraplastid events. Chlorophyll degradation occurs *via* the pheophorbide *a* oxygenase /phyllobilin (PaO/PB) pathway comprising two steps occurring respectively inside and outside the plastid. Degradation of phototoxic free Chl molecules and their intermediates occurs at the thylakoid membrane and is followed by modifications of Chl catabolites (reviewed in Kuai *et al.*, 2018; Chen *et al.*, 2016). In Arabidopsis leaves during senescence, chlorophyll *b* degradation is the first step in the degradation of LHCII and the thylakoid membrane, which is regulated by NON YELLOW

COLORING 1 (NYC1), but how the LCH are dismantled is poorly understood. During seed maturation, most of the mRNAs and proteins associated with the photosynthetic function decrease whereas the components of the plastid transcriptional machinery increase (Allorent *et al.*, 2013; Liebers *et al.*, 2017).

In *Arabidopsis*, blocking the photosynthetic linear electron transport during maturation results in reduced longevity (Allorent *et al.*, 2015). Chl retention in dry mature seeds is often linked to reduced seed longevity and poor germination, but the cause-effect relationship remains elusive (Clerkx *et al.*, 2003; Nakajima *et al.*, 2012; Fang *et al.*, 2014; Li *et al.*, 2017; Allorent *et al.*, 2015; Dekkers *et al.*, 2016). In *Arabidopsis*, mutants with defects in both NYC1 and NYC1-LIKE (NOL) produced seeds with reduced longevity and 10-fold more Chl compared to wild-type (Nakajima *et al.*, 2012). Chl retention in the *nye1 nye2* double-mutant (NON-YELLOWING (NYE), being responsible for Chl *a* degradation, and in the soybean *d1 d2*, a green seed mutant defective in *NYE/STAY GREEN*, caused a rapid loss in germination when seeds were stored in continuous light (Li *et al.*, 2017). Yet, there is no report from seed banks that green pea seeds harbouring a mutation in the *SGR* gene are sensitive to seed ageing. Likewise, in *Arabidopsis*, seeds of the double mutant *sgr1 sgr2* did not show altered germination before and after storage (Delmas *et al.*, 2013). Therefore, the association of Chl retention with decreased longevity remains to be explained.

Both Chl degradation and induction of seed longevity during maturation are regulated by ABA signalling. ABI3 is a transcriptional activator of *SGR* in *Arabidopsis* seeds (Delmas et al., 2013). In legume seeds, ABI5 is a positive regulator of seed longevity, dormancy and storage proteins (Zinsmeister *et al.*, 2016). It acts in a genetic network with ABI3 and other genes such as *DELAY OF GERMINATION 1* to regulate longevity-specific LEA genes, heat shock proteins and oligosaccharide accumulation (Righetti *et al.*, 2015; de Souza Vidigal *et al.*, 2016; Dekkers *et al.*, 2016; Zinsmeister *et al.*, 2016). ABI5 and additional ABA-RESPONSIVE ELEMENT-BINDING FACTOR (ABF) are also part of the regulatory network of Chl breakdown by controlling the expression of several genes coding Chl catabolic enzymes (Nakajima *et al.*, 2012; Dekkers *et al.*, 2016). In legumes, seeds of *abi5* retained some Chl and exhibited decreased longevity (Zinsmeister *et al.*, 2016). However, in these seeds, the remanence of Chl could not be explained by a deregulation of genes implicated in Chl synthesis or degradation. Intriguingly, mutant *abi5* seeds exhibited deregulated photosynthesis associated genes (PhANGs), suggesting a possible genetic link between longevity and photosynthesis (Zinsmeister *et al.*, 2016).

Gene co-expression analyses during maturation in seeds of *Medicago truncatula* and tomato identified ABI4 as a transcription factor correlated with seed longevity (Bizouerne *et al.*, 2021; Verdier *et al.*, 2013). ABI4 is a versatile enhancer of the ABA signal transduction during development and in response to stress. ABI4 positively regulates dormancy by mediating ABA synthesis and gibberellin (GA) degradation (Shu *et al.*, 2013; Shu *et al.*, 2016; Cantoro *et al.*, 2013) and negatively regulates germination by repression of cytokinin downstream response regulators (Huang *et al.*, 2017). In seedlings, ABI4 is also important for post-germinative growth in response to light, being implicated in cotyledon greening (Shu *et al.*, 2013; Xu *et al.*, 2016), repression of shade-induced hypocotyl elongation in response to damaged chloroplast (Ortiz-Alcaide *et al.*, 2019). It is also involved in sugar-induced repression of PhANGS (Acevedo-Hernández *et al.*, 2005; Penfield *et al.*, 2006). *In vitro,* ABI4 activates ABI5 through binding (Bossi *et al.*, 2009). Comparison of ABI4 and ABI5 gene targets shows that both transcription factors interact synergistically when ectopically expressed in leaves (Reeves *et al.*, 2011). However, the role of ABI4 during seed maturation is poorly understood.

This study aims at investigating whether ABI4 is part of the regulatory network controlling seed longevity in *M. truncatula*. Seeds of *Mtabi4* mutants exhibited both a severely decreased longevity and a green seed phenotype. *Mtabi4* mutant seeds were used as a tool to

gain further knowledge into the dismantlement of light-harvesting complexes (LHC) during degreening. We show that ABI4, in concert with ABI5, is required to coordinate eoplast formation and Chl degradation during seed maturation, which otherwise impedes the acquisition of longevity.

RESULTS

MtABI4 regulates seed vigor

To investigate the role of MtABI4, *Tnt1* insertion mutants were obtained with insertions located within the AP2 domain, 399 and 442 bp downstream the start-codon, respectively (Figure 1a). Germination in the dark of after-ripened seeds showed that both *Mtabi4* alleles exhibited reduced sensitivity to ABA (Figure 1b), like *abi4* mutants of *Arabidopsis* (Finkelstein *et al.*, 1998). To confirm the orthology of MtABI4 with ABI4 of Arabidopsis, the *abi4-1* mutant of *Arabidopsis* was transformed with *MtABI4* and *AtABI4* as a control, both under the Arabidopsis *ABI4* promoter. MtABI4 complemented the *Arabidopsis* mutant for ABA insensitivity as over-expressing seeds germinated to similar levels as WT and *pABI4*::*AtABI4* seeds in the presence of 2 µM ABA (Figure 1c).

To assess the impact of the *Mtabi4* mutation on seed development, seed filling and loss of water content during maturation were monitored. No significant difference in these traits was observed between wild-type and mutant seeds (Figure S1). Total time of maturation until pod abscission was also comparable for all lines, being around 44 d after pollination (Figure S1). To test if dormancy was impacted in the *Mtabi4* seeds, freshly harvested seeds were imbibed in the dark and in the light, since in this species light enhances dormancy (Bolingue *et al.*, 2010). In darkness, mature *Mtabi4* seeds germinated 1.5-fold faster than WT, whereas in the light, mutant seeds germinated 2-fold faster than the WT (Figure 1d). After-ripening for 2 months led to an increase in germination speed for all genotypes but faster germination was still visible for the mutants (Figure S2). These results indicate that MtABI4 is implicated in seed dormancy like in Arabidopsis.

In *Arabidopsis*, *abi4* seedlings showed defects in hypocotyl elongation during de-etiolation (Xu *et al.*, 2016). Here, MtABI4 was also found to increase hypocotyl elongation during de-etiolation when seeds that germinated in the dark were transferred to low light intensity, since hypocotyls of the *Mtabi4* mutants were much shorter than the WT after transfer (Figure 1e). As a proxy of seed vigor, a controlled deterioration test was carried out and showed that the loss of germination during incubation at 42°C 100%RH was much faster in mutant seeds (Figure S3). These results show that MtABI4 is an important regulator of physiological seed traits in the legume *M. truncatula*.

Mtabi4 seeds exhibit decreased longevity and a green seed phenotype

Since inference from gene co-expression analyses suggested that *MtABI4* was linked to longevity (Verdier *et al.*, 2013; Bizouerne *et al.*, 2021), longevity of the *Mtabi4* seeds was determined by monitoring the loss of germination of after-ripened seeds during storage at 75% relative humidity (RH), 35°C. Seed life span seeds was reduced by 75% in *Mtabi4* compared to wild type (WT) and associated WT seeds (Figure 2a). Loss of viability of the non-germinating mutant seeds was confirmed after 15 d of ageing by the complete absence of tetrazolium staining (Figure S4). Mutant seeds that were kept on the bench (60-65% RH, 20-22°C) showed a reduction in germination from 99% to 68% after one year, and even storage at 4°C in laminated bags did not prevent the seeds from ageing: they exhibited a decrease to 60% in viability after two years of storage compared to WT (data not shown). In mature Arabidopsis *abi4* seeds, longevity was also reduced compared to Col-0 seeds when stored at 75%RH, 35°C,

albeit to a lesser extent than observed for Medicago (reduction of 17% compared to WT, Figure S5a).

Another phenotype observed in developing *Mtabi4* seeds was a delay in the degreening process (Figure 2). Loss of chlorophyll started at 24 days after pollination (DAP) in WT seeds but over 10 d later in mutant seeds (Figure 2b). At 32 DAP, *Mtabi4-1* seeds contained 3-fold more Chl (Figure 2c) and mature, dry *Mtabi4* seeds retained 8-fold more Chl compared to WT (Figure 2d). To understand why degreening was impaired in *Mtabi4* seeds, a transcriptome analysis during maturation was performed at the onset of seed filling (13 DAP), midway through seed filling and before the induction of longevity (24 DAP), and in mature seeds (>44 DAP) (Figure 2e). Transcript levels of most of the genes coding enzymes involved in Chl synthesis were not affected in developing mutant seeds, including transcripts of key genes coding enzymes involved in the rate limiting step of tetrapyrrole biosynthesis, such as glutamyl-tRNA reductase (*MtHEMA*) and glutamate 1-semialdehyde aminotransferase (*MtGSA*) (Solymosi and Schoefs, 2010). Only transcripts of the light-dependent protochlorophyllide oxidoreductase A (*MtPORA*), the primary enzyme catalyzing protochlorophyllide (Pchlide) photoreduction, were higher in dry *Mtabi4* seeds. The difference in the degreening process in *Mtabi4* mutants compared to WT can also not be explained by deregulation of genes associated with Chl degradation (Figure 2e, Figure S6). In different species, mutants in *NYC1*, *NOL* and *SGR* produce green seeds (Delmas *et al.*, 2013; Nakajima *et al.*, 2012; Zhou *et al.*, 2011). However, in the *Mtabi4* mutants, transcripts of *MtNYC1*, *MtNOL* and *MtSGR* were not different from the WT, increasing from 24 DAP onwards (Figure S6).

It is noteworthy that *GUN4* and *GUN5*, coding two multifunctional proteins associated with chloroplast signaling and regulation of Chl synthesis (Solymosi and Schoefs, 2010), were deregulated in *Mtabi4* seeds (Figure 2f, g). In WT, their transcript levels decreased strongly throughout seed development. Such decrease was delayed in mutant seeds, since at 24 DAP, *GUN4* and *GUN5* transcript levels were four-fold higher in *Mtabi4* seeds than WT (Figure 2f, g). A similar observation was made for *BALANCE of CHLOROPHYLL METABOLISM (MtBCM1,* Figure 2h). In *Arabidopsis*, BCM1 is a key regulator of Chl homeostasis *via* binding to GUN4 (Wang *et al.*, 2020; Yamatani *et al.*, 2022). These data suggest that Chl homeostasis was possibly affected in the *Mtabi4* seeds, but not via the direct deregulation of Chl biosynthesis or degradation pathways.

Chlorophyll retention in *Mtsgr* mutant seeds is not associated with longevity

To further investigate a possible cause-effect relationship between reduced longevity and Chl retention, the *Mtsgr* mutant that retains Chl at high levels was studied (Zhou *et al.*, 2011). Visual inspection of mature seeds revealed that the green seed phenotype of this mutant was comparable to that of *Mtabi4* seeds (Figure S7a). 77K fluorescence spectroscopy showed that dry *Mtsgr* seeds presented an emission band at 682-684 nm, reflecting the presence of Chl-protein complexes typical of PSII but probably not associated to the reaction centre (Figure S7b) (Schoefs *et al.*, 1992). The weak fluorescence in the range of 700-760 nm corresponds to Chl(ide) secondary fluorescence bands and possibly to traces of PSI. In comparison, using the same normalization settings, the *Mtabi4* mature seeds did not show any particular feature that was comparable to *Mtsgr* seeds, demonstrating that seeds of both genotypes store Chl in different pigment-protein complexes.

Longevity was determined on freshly harvested seeds of WT and *Mtsgr* plants. In contrast to *Mtabi4* seeds, viability during storage was maintained even longer in the *Mtsgr* seeds than the WT (Figure S7). These data demonstrate that Chl retention *per se* does not cause a reduced longevity in *M. truncatula*. Likewise, in *Arabidopsis*, there was no difference in Chl content in *abi4* seeds compared to WT and yet longevity was reduced (Figure S5). Altogether,

this suggests that other events that are indirectly related to Chl degradation must be responsible for the decreased longevity in *abi4* mutant seeds.

MtABI4 and MtABI5 act in synergy to regulate longevity and degreening

In legume seeds, ABI5 regulates both the acquisition of seed longevity and degreening during late seed maturation (Zinsmeister *et al.*, 2016). These phenotypic similarities with *Mtabi4* prompted us to investigate if both transcription factors operate in the same pathway or act synergistically. Transcriptome comparison at 24 DAP between *Mtabi5* (Zinsmeister *et al.*, 2016) and *Mtabi4* seeds revealed an overlap of only 17 and 23% of genes that were respectively up and down differentially expressed in both genotypes (Figure 3a), with no significant GO enrichment. In contrast, in mature dry seeds, 50% of the up-regulated transcripts in *Mtabi5* seeds were in common with the *Mtabi4* transcriptome (Figure 3a). This list of common genes was enriched in GO terms associated with "response to ABA" and genes coding proteins located in "plastid" and "thylakoids" (Table S2). Likewise, among the genes that were down-regulated in mature seeds of both mutants, several GO associated with photosynthesis were found (Table S2). This suggests that both MtABI4 and MtABI5 regulate genes associated with photosynthesis during the second half of seed maturation, when Chl is degraded.

To further investigate the pathways in which both transcription factors are implicated, a double mutant *Mtabi5-2 Mtabi4-1* was created and seeds were produced to examine longevity and degreening. The life span of this double mutant during storage at 75% RH, 35°C was much shorter than the respective single mutants (Figure 3b). While time to 50% loss of viability (P50) was reduced to 80% and 50% in *Mtabi4* and *Mtabi5* mutant seeds, respectively, this reduction was 92% in the double mutant compared to WT, suggesting that both transcription factors acted via independent pathways. A synergistic interaction between both transcription factors was also observed for the Chl content in mature seeds, with amounts 5-fold higher in the double mutant compared to *Mtabi5* seeds and 1.7 fold higher than in *Mtabi4* seeds (Figure 3c, d). We also examined the effect of both transcription factors on the *PhANG* transcript levels in dry seeds. *PhANGs* are known *in vitro* targets of ABI4 (Acevedo-Hernández *et al.*, 2005) and their transcript levels strongly decrease during maturation in Arabidopsis (Allorent *et al.*, 2013). In developing and mature *Mtabi5* seeds, we previously discovered that *PhANG* transcripts remained higher compared to WT seeds (Zinsmeister *et al.*, 2016). Using four selected genes representative of PSI and PSII (Figure 3e-h), *PhANG* transcripts were confirmed to be more abundant in dry single mutant seeds compared to WT (Figure 3e-h), although transcript levels remained much higher in *Mtabi5* seeds compared to *Mtabi4* seeds. In the double mutant seeds, the transcript levels were identical or slightly higher (*MtLHCA1*, *MtPsbW*) than those of *Mtabi5* seeds. This suggests that MtABI5 could be more important than MtABI4 in regulating PhANGs transcript levels during late maturation.

Since MtABI5 also regulates the synthesis of protective mechanisms that play a role in longevity and considering the synergistic role of MtABI4 with MtABI5 in degreening and longevity, we examined whether MtABI4 was also implicated in the same protective pathways via the study of two marker genes, Mt*EM1* and *SEED IMBITION PROTEIN 1* (MtSIP1, a raffinose synthase, Chatelain *et al.*, 2012; Righetti *et al.*, 2015; Zinsmeister *et al.*, 2016). For both genes, no difference in transcript levels was found in mature *Mtabi4* seeds compared to WT (Figure 3i, j), suggesting that these pathways are not under the regulation of MtABI4.

Mtabi3 mutant seeds exhibit both a green phenotype and desiccation sensitivity (Delahaie *et al.*, 2013), and a deregulation of *MtABI3* might explain the MtABI4 genotype. Throughout development (Figure S) and in mature seeds, the transcript level of *MtABI3* in *Mtabi4* seeds was lower compared to WT seeds (Figure 3k), whereas it was identical in *Mtabi5* and *Mtabi4 Mtabi5* seeds. However, known ABI3 targets, such as *MtSGR* (Figure S6, Lalanne

et al., 2021) or *MtEM1* (Figure 3i) were not different in the *Mtabi4* seeds compared to the wildtype, suggesting that the *Mtabi4* phenotype was not caused by deregulation of MtABI3.

Considering that in *Arabidopsis,* ABI4 regulates ABI5 by binding to its promoter (Bossi *et al.*, 2009), we investigated if these transcription factors regulate one another in *M. truncatula* seeds. The RNAseq data of *Mtabi4* seeds during maturation showed no differential expression of *MtABI5* (Table S1). *MtABI5* expression was also not deregulated in the dry mature seeds of *Mtabi4* mutants (Figure 3). In contrast, expression level of *MtABI4* was significantly reduced by 20% in mature *Mtabi5* seeds compared to WT (Figure 3l). To further investigate whether MtABI5 could regulate *MtABI4* expression in photosynthetic tissues, *MtABI5* was ectopically expressed in *M. truncatula* leaves using a 35S::*MtABI5*/35S::*GUS* construction and activated by ABA. A known target of ABI5, *MtEM1,* was used as control (Zinsmeister *et al.*, 2016). Over-expression of *MtABI5* led to a 10-25-fold increase in *MtABI4* transcript level compared to leaves over-expressing the control plasmid (Figure 3m) whereas the increase in *MtEM1* transcript level was 5-fold. Altogether, this suggests that MtABI4 and MtABI5 act *via* dependent and independent pathways to regulate seed maturation and acquisition of longevity.

In *Arabidopsis*, ABI4 is an essential element of the sugar signalling pathway (Bossi *et al.*, 2009; Acevedo-Hernández *et al.*, 2005) whereas in legumes, ABI5 activates the synthesis of RFO which typically accumulates at the end of seed maturation and is necessary for longevity (Righetti *et al.*, 2015; Chatelain *et al.*, 2012). No synergistic effect of MtABI4 and MtABI5 was found for sucrose and RFO levels (Figure S8). Sucrose content was only impacted in *Mtabi5* seeds compared to wild-type. Stachyose content, which represents 90% of the total RFO was reduced by half in all three mutants, suggesting redundant regulatory pathways (Figure S8). Levels of hexoses, which usually disappear during maturation drying, were higher in *Mtabi4* seeds and in the double mutants compared to WT and *Mtabi5* seeds. The increase in glucose could be an indirect effect of the Chl retention that could still stimulate photosynthesis.

MtABI4 regulates eoplast formation during maturation

The transcriptome of developing *Mtabi4* seeds was further examined to understand why longevity was impaired. The number of deregulated genes increased from 908 at 13 DAP to 7522 in dry seeds, suggesting that MtABI4 is important for the late seed maturation program (Figure 4a). Yet, GO enrichment analysis revealed that the first symptom of perturbation in developing *Mtabi4* seeds was found as early as 13 DAP, with an enrichment of down-regulated genes involved in regulation of photosynthesis biosynthetic processes (Figure 4c). Later during seed maturation, an increasing number of over-represented GO terms were associated to functions related to thylakoids and photosynthesis in the mutants. Over 90 nuclear and plastid genes associated with chloroplast functions were deregulated in the developing *Mtabi4 s*eeds, including PhANGs. Here, their transcript levels declined strongly between 13 and 24 DAP in WT seeds (Table S1, Figure 4b, c). In contrast, such decrease was delayed in the mutant seeds at 24 DAP (Figure 4b, c).

Considering that the decrease in PhANG transcripts is the predominant transcriptional change during eoplast formation and associated dismantling of the LHC (Allorent *et al.*, 2013), it might be hypothesized that MtABI4 plays a role in coordinating both phenomena. To test this hypothesis, we investigated whether such coordination was impaired in developing *Mtabi4* seeds using the transcriptome data. Conversion from one plastid type to another is accompanied by significant changes in plastid transcription (Liebers *et al.*, 2017). Plastidial genes are transcribed by two different types of RNA polymerases: one encoded by the plastid genome (PEP), which transcribes photosynthesis associated plastidial genes, PhAPG) and one encoded by nuclear genes (nuclear-encoded RNA polymerases, NEP). Establishment of a correct NEP/PEP configuration is absolutely essential for successful chloroplast differentiation

(Allorent *et al.*, 2013; Liebers *et al.*, 2017; Yoo *et al.*, 2019). If MtABI4 is important for the chloroplast-to- eoplast conversion, symptoms of a disturbed NEP/PEP ratio should be found in the transcriptome of mutant seeds. As a proxy for NEP/PEP ratio, we measured the ratio of transcript levels (mean normalized counts, Table S1) encoding PhAPGs and PhANGs. Ratios were 2- to 6-fold lower in *Mtabi4* seeds compared to WT both at 24 DAP and in dry seeds (Figure 5a). These data suggest the *Mtabi4* dysfunction led to uncoupled expression of PhANGs and PhAPGs during maturation.

Since the chloroplast functioning relies on both plastid- and nuclear encoded genes, retrograde signaling from the plastid to the nucleus is necessary to coordinate the transcription of photosynthesis genes (Ortiz-Alcaide *et al.*, 2019; Martín *et al.*, 2016; Wu and Bock, 2021). *MtGUN4* and *MtGUN5,* which were significantly deregulated in *Mtabi4* seeds (Figure 2e), are genes involved in retrograde signalling in *Arabidopsis* (Wu and Bock, 2021). Therefore, to investigate whether the retrograde signaling was affected at the transcriptional level in *Mtabi4* developing seeds, we compared their transcriptomes with an *Arabidopsis* seedling data set containing genes that were deregulated when retrograde signaling was activated after inhibition of chloroplast biogenesis by lincomycin (Martín *et al.*, 2016) . In *Mtabi4* seeds harvested at 24 DAP, there was a significant over-representation of genes that were down-regulated in *Arabidopsis* when retrograde signaling is activated (Figure 5b). Consistent with this, the orthologue of *GOLDEN LIKE 1* (*GLK1*) was also found deregulated in *Mtabi4* at all three stages of seed maturation (Figure S9). GLK1 is a master regulator of plastid retrograde signalling and a key gene in the control of PhANGs expression during chloroplast development and also transcriptionally regulates *BCM1* (Martín *et al.*, 2016; Yamatani *et al.*, 2022).

Additional evidence that *Mtabi4* is pivotal to eoplast formation was provided by electron microscopy and fluorescence spectroscopy. At 32 DAP, most cells of WT presented plastids containing internal membranes that were interpreted as remains of thylakoids (Figure 5c). In contrast, in *Mtabi4* seeds, thylakoids were still clearly apparent in most of the cells. These observations suggest that the dismantlement of the photosynthetic apparatus during maturation was faster in the WT than in the *Mtabi4* mutants. In mature seeds, following 6h of imbibition in dim light, *Mtabi4* mutants exhibit aberrant plastids characterized by a misformed prolamellar body with randomly convoluted membranes, whereas in WT seeds a typical paracrystalline structure with an array of prothylakoids was observed (Figure 5c, Solymosi and Schoefs, 2010).

To obtain further information on the configuration of the photosystems during maturation in Mtabi4 vs WT, 77K fluorescence spectroscopy was performed (Figure 6). The normalized spectra of all genotypes exhibited an intense peak around 726-730 nm, typical of PSI (Figure 6, Schoefs et al. 1991). At 24 DAP, no clear difference in PSI was observed between mutants and WT seeds (Figure 6a), but at 32 DAP, the dismantlement of the PSI appeared more advanced in the WT than in the mutants (Figure 6c, d). This was visible in the 2nd derivative spectra of the PSI band which generated a negative band (Figure 6d). In the WT, two components could be distinguished at 721 and 731 nm in the negative band, demonstrating a strong decrease in the energy transfer between PSI components, and a dislocation of the PSI components. In contrast, the negative band of the 32 d-old *Matbi4* seeds was symmetric with no particular feature (Figure 4d).

Next to the PSI fluorescence band, a broad peak was seen in the region of 620-680 nm. In green leaf, this peak encompasses fluorescence due to the PSII internal LHC (Figure S7, 685-695 nm, Schoefs *et al.*, 1992), the presence of Chl precursors (623-657 nm), free Chl(ide) (675 nm, Schoefs, 2001)) and non-integrated protein-Chl complexes (675-680 nm, Schoefs and Franck, 2008)). In seeds at 24 and 32 DAP, the relative fluorescence intensity of the 671-678 band was much weaker in *Mtabi4* seeds compared to WT (Figure 6a, c). In WT seeds at 24 DAP, the second derivative spectra revealed the appearance of a negative peak at 673 nm (Figure 6b), whereas this peak was no longer observed at 32 DAP (Figure 6d). This transient

appearance in the WT seeds is also visualized as a broad peak in the difference between the second derivative spectra of 24 and 32-d old seeds (Figure S10). In *Mtabi4* seeds, this negative peak in the second derivative spectra was observed only at 32 DAP (Figure 6d), indicating a delay compared to WT seeds. In the region of 620-680 nm, another component in the spectrum was found in the 32 d-old WT seeds in the form of a shoulder at 665 nm that was absent in the mutants (Figure 6c, insert). The presence of this 665 nm shoulder and the 671-678 nm band in WT seeds indicate respectively the presence of free Chl(ide) molecules and/or pigment-protein complexes that are no longer integrated in the light-harvesting antenna.

The presence of 77K fluorescence bands around 678 nm (Figure 6c, insert) could also be due to *de novo* Chl(ide) synthesis (Schoefs, 2001). We ruled out this interpretation because the light-minus-dark spectra at 32 DAP did not show any positive band after a flash light at -20°C in WT seeds (Figure 6f). Instead, a negative band around 665-668 nm was observed only in the *Mtabi4* seeds, after a flash light at -20°C. This suggests that the flash triggered Chl degradation through photooxidation and potential ROS formation. This observation represents a snapshot of the dismantling of the photosynthetic apparatus at 32 DAP. In dry seeds, the remaining Chl molecules were stored in the remaining PSI and PSII, being almost undetectable (Figure 6e). Altogether, this suggests that MtABI4 stimulates dismantling of the photosystems at an early stage during seed maturation that will lead to the formation of eoplasts in dry seeds.

To find out if MtABI4 and MtABI5 act in synergy in chloroplast dismantling, we compared the 77K emission and photosynthesis efficiency of the double mutant seeds with those of *Mtabi4* seeds. The *Mtabi5 Mtabi4* seeds behaved like *Mtabi4* seeds during development (Figure 6), indicating that the double mutant did not exhibit a further delay in chloroplast dismantling. Altogether this indicates that MtABI4 is mostly responsible for shut down of photosynthetic activity and inducing eoplast formation and while the degradation of Chl is performed in synergy with MtABI5.

Functional importance of timely eoplast formation on longevity

To investigate the functional importance of MtABI4 in dismantling PSI and PSII during eoplast formation, we first tested whether altering the light conditions during seed development had an impact of longevity and degreening in *Mtabi4* seeds. For this, plants were grown under normal light conditions until pods were formed. Then, some pods were wrapped in a black cloth until abscission, after which the seeds from control (light) and dark treatments were collected and tested for longevity. An ageing time point was chosen so that both control WT and *Mtabi4* seeds that were produced under control light conditions would give around 50% viability after storage (Figure 7). Growing pods in the dark did not have a negative impact on longevity of WT seeds (Figure 7a). In contrast, *Mtabi4* seeds that developed in the dark exhibited a significant increase in viability after ageing compared to mutant seeds grown on the same plant under light. This shows that light has a detrimental effect in the absence of a functional MtABI4. Darkness during maturation also triggered Chl degradation in *Mtabi4* seeds, as the colour of mature seeds appeared identical to WT (Figure 7b). This reinforces the conclusion that MtABI4 does not directly regulate Chl degradation.

Given the delay in LHC dismantling during maturation and that darkness restores longevity in *Mtabi4* seeds, we evaluated whether photosynthesis activity was affected in developing mutant seeds by assessing 298K Chl fluorescence kineticsat the PSII(Figure 7c). In WT seeds, Fv/Fm values decreased between 24 and 32 DAP from 0.6 to 0.3 (Figure 7c) and no electron transport could be detected in the dry state (Figure S11). This is consistent with previous observations on pea seeds showing PSII is progressively being dismantled during late seed maturation (Smolikova *et al.*, 2017). In *Mtabi4* seeds, Fv/Fm values were significantly higher at 32 DAP. The parameter Sm reflects the energy requested to close all the PSII through

reduction of Q_A and all the other electron transporters (Stirbet and Govindjee, 2011). In WT seeds, Sm values increased significantly between 24 and 32 DAP (Figure 7c), which indicates that the light harvesting capacity of PSII is decreasing during maturation (Figure 7d). In contrast, in 32 d-old *Mtabi4* seeds, the Sm value was significantly lower than in WT and comparable to those of 24 DAP. The parameter DF represents a photosynthesis performance index based on absorbed light, reaction center density, trapping efficiency and electron transport efficiency. DF values were significantly higher for *Mtabi4* seeds than for WT at 24 DAP (Figure 7e), indicating that the chloroplasts of mutant seeds were more efficient in performing photosynthesis than WT. Altogether, these data indicate that the dismantlement of PSII is delayed in mutant seeds compared to WT.

We next investigated whether the delayed shut-down in photosynthesis in developing *Mtabi4* seeds could generate an oxidative stress during maturation drying using the ROSWHEEL tool that provides sets of robust marker transcripts for specific perturbations that generate ROS (Willems *et al.*, 2016). In *Mtabi4* seeds harvested at 24 DAP, there was a significant over-representation of marker genes that were deregulated when ROS are generated by perturbations of the retrograde signaling (GUN retro, Figure 7f). Furthermore, the transcriptome of mature dry *Mabi4* seeds was enriched in marker genes associated with ROS generated, after UV-B treatment and early $^1O_2$-induced changes ($^1O_2$–UV-B ROS).

DISCUSSION

For chlorogenic embryos, seed maturation is associated with the loss of chlorophyll content that is regulated by the ABA signaling pathway, under the control of ABI3, ABI5 and ABF proteins (Delmas *et al.*, 2013; Nakajima *et al.*, 2012; Zinsmeister *et al.*, 2016; Dekkers *et al.*, 2016). This study provides an additional genetic link between degreening and longevity and establishes ABI4 as a new regulator acting in synergy with ABI5 to coordinate the dismantling of the photosynthesis apparatus and Chl degradation. Here, we demonstrate that ABI4 controls the induction of the chloroplast-to-eoplast conversion during seed maturation. Eoplast formation allows the shutting down of photosynthesis before the end of seed desiccation, thereby avoiding conditions that favour ROS formation when longevity is acquired during maturation drying. This study reinforces previous intriguing findings that the regulation of chloroplast evolution and photosynthesis within the embryo during maturation affects the development of the future plant (Allorent *et al.*, 2015; Sela *et al.*, 2020; Kim *et al.*, 2009). It also demonstrates the importance of ABI4 as a regulator of seed vigor.

Chl retention in *Mtabi4* and *Mtabi5 Mtabi4* seeds was associated with a decreased longevity (Figure 3), as previously shown for green seed mutants defective in Chl degradation in Arabidopsis (Nakajima *et al.*, 2012) and soybean (Li *et al.*, 2017). We ruled out that the remanence of Chl *per se* is the direct cause for decreased seed longevity for the following reasons: 1) in Arabidopsis *abi4* seeds, longevity was affected but Chl content was not (Figure S5); 2) mature green *Mtsgr1* seeds contained Chl-protein pigments and yet longevity was not impaired (Figure S7); 3) no free phototoxic Chl was found in dry seeds of any of the genotypes; 4) in dry tissues, there was no electron transport at the PSII as shown by the low Fv/Fm values. This absence of electron transport is explained by the low hydration levels in dry seeds and is consistent with previous observations on pea seeds (Vertucci *et al.*, 1985; Smolikova *et al.*, 2018) and green resurrection plants (Zia *et al.*, 2016). Furthermore, despite the presence of Chl in the *Mtabi4* mature seeds, no photosynthetic activity could be detected during the first 22 hours of imbibition (Figure S11). Therefore, it is unlikely that the remaining Chl and electron transport would generate ROS during storage leading to loss of viability; 5) seed ageing and germination following storage were performed in the dark. These conditions prevent the

excitation of the remaining Chl molecules in *Mtabi4* seeds that would generate ROS (Kim *et al.*, 2009; Pavel Pospíši, 2016) during storage and lead to increased deterioration.

A delayed dismantlement of the photosynthetic apparatus during seed maturation is an attractive hypothesis to explain the decreased longevity in *Mtabi4* seeds. It is supported by electron microscopy (Figure 5), 77 K fluorescence spectroscopy (Figure 5), 298 K fluorescence kinetics (Figure 7), transcriptome data (Table S1; Figure 4) and reversal of the longevity phenotype when mutant seeds were developed in the dark (Figure 7a). Such delay cannot be attributed to a general delay in seed development because the seed dry weight accumulation and maturation drying were identical between mutants and WT seeds (Figure S1) and the gene profiles belonging to GO terms associated with seed development did not exhibit a delay in *Mtabi4* seeds (Figure 4d). Also, *MtSIP1* and *MtEM1*, two markers of late seed maturation, did not show any delay in their expression pattern compared to WT (Figure 3).

The 77 K fluorescence measurements performed during the second half of maturation provides a scenario of the dismantlement of the photosynthetic apparatus by comparing WT and mutant seeds. In the WT, the dislocation of LHC starts as early as 24 DAP by the appearance of free Chl-protein complexes. Between 24 and 32 DAP, the antenna of LHC1 becomes partially dislocated as evidenced by the heterogeneity of second derivative spectrum at 721 and 731 nm (Figure 6 and Figure S10). This is reminiscent of the dislocation of PSI in red algae (Doan *et al.*, 2003). Simultaneously, free Chl-protein complexes keep being formed while releasing Chl molecules, which leads to the formation of pool of free pigments as evidenced by the shoulder peak at 665 nm. In parallel, Chl content decreases over two-fold (Zinsmeister *et al.*, 2016). These components are then degraded during maturation drying as no Chl fluorescence could be detected in dry seeds. In *Mtsgr* seeds that remained green at maturity but for which Chl ended up in different pigment-protein complexes, longevity was not affected (Figure S7).

The presence of free Chl and free Chl-protein complexes are potentially harmful as they increase the potential to form ROS under light. The delay in LHC dislocation observed at 32 DAP in the *Mtabi4* and double mutant could favor ROS formation when seeds start to desiccate during maturation drying, between 40 and 44 DAP (Figure S1b). Such hypothesis is supported by the observation that a flash triggered Chl degradation in the *Mtabi4* seeds at 32 DAP through photooxidation and potential ROS formation, in contrast to WT seeds (Figure 6f), as well as the transcriptome data showing significant footprints of ROS formation (Figure 7f). Consistent with this, several detoxification genes were 2-fold over-expressed in dry mature *Mtabi4* seeds such as homologues of *CHLOROPLAST ALDEHYDE REDUCTASE, 1-CYS-PEROXIREDOXIN* and *FERRITIN 2*. These genes were also found to be deregulated when *ABI4* was ectopically expressed in *Arabidopsis* seedlings (Reeves *et al.*, 2011). In *Arabidopsis* seedlings, ABI4 is involved in photooxidation during detiolation *via* accumulation of free PChlide and Chlide which produced photo-oxidation and ROS, leading to cell death (Xu *et al.*, 2016). Here PChl(ide) was not detected but Chl(ide) accumulated during seed maturation as a result of the degradation of Chl-protein complexes of the photosynthetic apparatus was evidenced. We also showed that the photosynthesis efficiency was higher in immature *Mtabi4* and *Mtabi4 Mtabi5* seeds than in WT (Figure 7), which would also contribute to the production of a higher amount of ROS. Therefore, it is conceivable that to avoid a deleterious maturation drying, including by ROS formation, a coordinated dismantling of the photosynthesis apparatus must be switched on under the control of MtABI4. This hypothesis is further supported by previous work showing that disruption of photosynthesis during seed maturation has repercussions on seed ageing during storage (Allorent *et al.*, 2015). Indeed altering electron transport between PSII and I in Arabidopsis developing seeds using DCMU or mutants defective in the photosynthetic electron transport chain leads to decreased tolerance to deterioration during storage, which was also attributed to an oxidative stress (Allorent *et al.*,

2015). The observation that maturation of *Mtabi4* seeds in darkness improved longevity compared to seeds developed in normal light condition is consistent with these observations (Figure 7). In lettuce, the loss of seed viability during storage at high RH and temperature was also dependent on the light regime during seed development (Contreras *et al.*, 2009).

Our work shows that MtABI4 and MtABI5 play a synergistic role in seed longevity and Chl retention, suggesting that they act at least partially *via* independent pathways during maturation. This is consistent with previous observations showing that only 12% of ABI-transcriptional targets were induced by both transcription factors when they were ectopically expressed in seedlings and by yeast one-hybrid assays (Reeves *et al.*, 2011). How MtABI4 and MtABI5 control the dismantlement of LHC during maturation remains to be assessed. In Arabidopsis, mRNAs and proteins of components of the plastid transcriptional apparatus increase during the period of plastid dedifferentiation (Allorent *et al.*, 2013). In Arabidopsis leaves, ABA affects the transcription of chloroplast genes by a PP2C-dependent activation of nuclear genes encoding proteins involved in chloroplast transcription (Yamburenko *et al.*, 2015). The disturbed changes in PEP/NEP transcripts ratio in developing *Mtabi4* seeds (Figure 5) is consistent with these observations. However, the PEP/NEP ratio was not affected in *Mtabi5* seeds during maturation (Zinsmeister *et al.*, 2016), suggesting the implication of a distinct ABA signaling pathway depending on MtABI4. GO enrichment analysis of *Mtabi4* and *Mtabi5* transcriptomes of seeds at 24 DAP did not reveal common terms between mutants but several GO functions and cellular processes associated with photosynthesis and plastids were found in dry seeds of each mutant (Table S2). PhANGs transcripts were deregulated in both mutants, albeit more strongly in *Mtabi5* seeds (Figure 3). In Arabidopsis seedlings, PhANGs expression is regulated positively by GLK1 (Waters *et al.*, 2009; Martín *et al.*, 2016). Throughout *M. truncatula* seed development, *MtGLK1* transcript levels were disturbed in *Mtabi4* mutants, being lower at 13 and higher at 24 DAP compared to WT seeds (Figure S9). Out the 93 genes that were induced by overexpressing GLK1 in Arabidopsis seedlings (Waters *et al.*, 2009), 42% (32) were also up-regulated in 24-d *Mtabi4* seeds, including homologous genes encoding several chlorophyll binding proteins (Table S3), a SGR-Like protein and BCM1. These genes were also deregulated in *Mtabi4* seeds (Figure 2). Also, some interaction between both transcription factors can be expected, since *MtABI4* expression during maturation was deregulated in *Mtabi5* seeds and is activated by *MtABI5* when ectopically expressed in leaves (Figure 3l, m) (Table S1). Altogether this points to a complex regulation of both nuclear and plastidial genes at the transcriptional and post-transcriptional level that are dependent on ABA signaling and progress of seed maturation. Zinsmeister et al. (2016) showed that the synthesis of protective molecules necessary for longevity was under the control of MtABI5. Here, we did not find evidence for such a role could be played by MtABI4. To summarize, Figure 8 shows a model where MtABI4 controls chloroplast-to-eoplast differentiation early during seed maturation to ensure coordinated Chl degradation and shutdown of photosynthesis, thereby avoiding cellular damage that would jeopardize seed longevity. In this model, MtABI4 and MtABI5 act in parallel pathways to induce longevity. Thus, the synergistic effect on longevity in the *Mtabi4 Mtabi5* mutant might be explained by the absence of MtABI5-dependent synthesis of LEA proteins and non-reducing sugars and the delay in MtABI4-controlled eoplast formation.

EXPERIMENTAL PROCEDURES

Methods

Plant material

Medicago truncatula ssp. tricycla (R-108) plants were cultivated in a climate chamber according to Chatelain et al. (2012). Flowers were tagged at pollination. Two independent *Tnt1* insertion lines in the *MtABI4* gene (MtrunA17_Chr5g0437371) were obtained from the Samuel Robert Foundation (Oklahoma, USA) and crossed to obtained homozygous lines: NF19813, referred to as *Mtabi4-1* and NF2525, *Mtabi4-2*. *Tnt1* insertions were verified by PCR using forward and reverse primers listed in Table S4. Homozygous *Mtabi5-2* and *Mtsgr* mutants were obtained from Zinsmeister et al. (2016) and Zhou et al. (2011), respectively. Double mutants *Mtabi5-2 Mtabi4-1* seeds were obtained by crossing *Mtabi4-1* with *Mtabi5-2* as a mother plant and screening off-springs for double homozygous mutants, taking advantage of a preliminary observation during our crossings that all homozygous Mt-*abi4-1* seeds were green regardless of the *MtABI5* segregation. Green seeds were subsequently screened for double homozygous mutants using PCR analysis (Table S4). To allow seeds to mature in darkness, 5-10 young developing pods (10-15 DAP) per plant were carefully wrapped in a black cloth and then covered in aluminum foil until abscission. After harvest, pods were dried in dim light at 44% RH, seeds were dissected and stored at -80°C until use. Homozygous lines of Arabidopsis *abi4* mutant (CS8104, Col-0 background) were ordered from Nottingham Arabidopsis Stock Center.

Complementation of MtABI4 in *abi4* mutants of Arabidopsis

MtABI4 (MtrunA17_Chr5g0437371) and *AtABI4* (At2g40220) were cloned from the full-length gDNA obtained from retro-transcription and RNA of mature seeds of WT R108 and Col0, respectively, using primers listed in Table S4. The coding region of MtABI4 was amplified by PCR and inserted into the vector pDONp5p2, while an AtABI4 promoter sequence of 3 kbp was inserted into pDONp5p1. The constructions AtABI4p::AtABI4 and AtABI4p::MtABI4 were transferred to the pKGW vector, and the identity of the insert was checked by sequencing. *Arabidopsis abi4* plants were transformed via *agl1* Agrobacterium-mediated gene transfer by floral dipping. Harvested seeds from transformed plants were sterilized and sown on selective medium (quarter-strength Murashige & Skoog, 0.8 % agar, 75 µg/mL kanamycin) and after two weeks seedlings were screened by PCR (Table S4). Independent single-insertion lines with the highest *MtABI4* expression were chosen for physiological characterization.

Ectopic over-expression of *MtABI5*

35S::MtABI5::GUS and the control (plasmid containing the GUS gene alone) plasmids were obtained by the gateway cloning method according to the manufacturer instructions (Directional TOPO® Cloning kits, Invitrogen) and used for *agl1 Agrobacterium* transformation. A 1,8 kb amplicon of MtABI5 and a GUS amplicon were obtained using primers listed in Table S4 and cloned in pK7WG2D,1 containing a 35S cauliflower mosaic virus promoter and GUS reporter that was used as transformation marker. Inserted sequences were verified by sequencing. The transformation protocol was according to Picard et al. (2013) with the following modifications: 1 ml of 10% (v:v) solution of 24h-old Agrobacterium culture was injected using a syringe in five replicates of three pooled *M. truncatula* leaves originating from 21d-old seedlings. Transgenic plants were grown for 24h at 22°C, 16h light. Thereafter, a 0.5 µM ABA solution was injected into the leaves which were incubated for an additional 48h than frozen in liquid N2 and stored at -80°C until RNA extraction as described below.

Physiological and biochemical assays

For germination assays, seeds of *M. truncatula* were scarified by sand paper and imbibed on Whatman filter paper with 4 mL of distilled water at 20°C in the dark or in continuous light in water or in the presence of indicated concentrations of ABA (mixed isomers, Sigma, St Louis, MO, USA). ABA was dissolved in methanol prior to dilution in water. Control seeds were imbibed in the MeOH concentration corresponding to the highest ABA concentration (0.05%

MeOH). For Arabidopsis, triplicates of 100 seeds pooled from at least 3 plants were imbibed in water at 20-22°C in continuous light. Mean germination time was calculated as $\sum(n \times d) / N$, where n = number of seeds germinated on each day, d = number of days from the beginning of the test, and N = total number of seeds germinated at the termination of the experiment. For seed longevity assays, mature scarified seeds were stored at 35°C in the dark at 75% RH using hermetically closed containers containing a saturated NaCl solution. At different storage time intervals, seeds were imbibed as described above, and germination percentage was counted after 10 d. Aged seeds were also incubated in a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride at 20°C for 18 h in the dark. Stained tissues were considered viable, and unstained white tissues were considered dead. Controlled deterioration test was performed by incubating mature scarified seeds at 40°C 100% RH and germination was tested as described above.
Chlorophyll and sugar contents were assayed by spectrophotometry and HPLC, respectively as described in Zinsmeister et al. (2016).

Chlorophyll fluorescence

Three replicates of 3-5 seeds were harvested during maturation and left on wet filter paper in the dark until measurements. After removing the seed coat in safe green light, embryos were directly used for PAM measurements according to (Yin *et al.*, 2010) or snap frozen in liquid nitrogen for 77 K fluorescence according to Schoefs et al. (2000).

RNA sequencing, quality control and reads alignment

Three biological replicates of 30 (RT-PCR) and 50 (RNAseq) seeds were harvested during maturation at 13 DAP and 24 DAP (WT and *Mtabi4-1*) and at maturity (WT, *Mtabi4-1* and *Mtabi4-2*) and frozen in liquid nitrogen. Total RNA was extracted using the NucleoSpin® RNA Plant Plus kit (Macherey-Nagel) according to the manufacturer's instructions. For RT-qPCR, residual gDNA was digested using 200 U of DNase I RNase free (Thermo Scientific™). Total RNA from high quality samples (RIN values > 8.9 evaluated by a 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) were used for library preparation sequencing. RNA-seq dataset was collected from single-end sequencing of cDNA libraries with 50 bp reads (mature seeds) and paired-end, 100 bp reads (13 DAP, 24 DAP seeds) using the BGISeq500 platform. Raw reads were filtered to remove adapters and low-quality reads, then mapped to the *M. truncatula* reference genome Mt5.0 (https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/ (Pecrix *et al.*, 2018). Total mapped reads and number of transcripts (counts and TPM) were estimated using the Salmon algorithm v.1.2 (https://combine-lab.github.io/salmon/). Differential expressions of genes between standard and heat stress conditions were calculated using the DEseq2 algorithm. DEG threshold was set as adjusted p-values below 5% following the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Over-representation analyses of Gene Ontology were performed using the ClusterProfiler package (version 1.3.1073) applying an adjusted p-value cutoff of <0.05 obtained after a Benjamini-Hochberg correction.

Real time quantitative PCR

One mg total RNA was reverse-transcribed using the iScript RT Supermix for RT-qPCR (Bio-Rad Laboratories), 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 *MtACTIN11* and *MtTCTP* (Zinsmeister *et al.*, 2016). Forward and reverse primers used are listed in Supplemental Table 4. Relative expression levels were calculated using the comparative $2\Delta(Ct)$ method.

Transmission electron microscope

Dried 32 days-old and mature seeds were imbibed for 6 h in the dark to allow the fixative to enter the tissue then cotyledons were isolated and incubated overnight at 4°C under vacuum in 2.5% (v/v) glutaraldehyde and 2.5 (w/v) paraformaldehyde diluted 50 mM phosphate buffer (pH 7.2). After rinsing with the phosphate buffer, samples were dehydrated in successive ethanol baths (50, 70, 95 and 100%) and embedded in Epon resin. Three to five embedded individuals of each genotypes were cut into 60-nm sections with an ultramicrotome equipped with a diamond knife. The sections were post-stained for 15 min with 3% uranyl acetate in 50° ethanol and observed using a JEOL JEM 1400 electron microscope.

Statistical analysis

All data were analyzed using three to five biological replicates retrieved from seed lots originating from 3-6 plants/genotype. Significant values on normally distributed data were determined using t-test or ANOVA test ($p<0.05$) and post hoc Tukey for multiple comparisons. For non-parametric data, a Kruskal-Wallis test ($p<0.05$) with post hoc Dunn test were used instead. Statistical over-representation was calculated using a hypergeometric probability calculator available at http://nemates.org/MA/progs/representation.stats.html.

Accession numbers

Sequence data in this study can be found under the following accession numbers MtABI5, MtrunA17_Chr7g0266211; MtABI4, MtrunA17_Chr5g0437371; MtSGR MtrunA17_Chr5g0398211. Arabidopsis Genome Initiative locus identifier for ABI4 mentioned in this article is At2g40220.

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AUTHORS CONTRIBUTION

OL and JB conceived the project, supervised the experiments and wrote the article with contributions from JZ, DL and BS. All authors revised the manuscript. JZ, DL, DTT, BLV performed the physiological and molecular experiments. BS and JM performed the fluorescence kinetics experiments. JZ, DL, BS, DTT, JB, JM and OL analysed the data.

DATA AVAILABILITY STATEMENT

All relevant data can be found in the manuscript and its supporting information. The raw RNAseq data obtained in this study have been deposited in the GEO database as Accession GSE212927.

CONFLICT OF INTEREST

The authors of this article have no conflict of interest to declare.

SUPPORTING INFORMATION

Supporting Figures:

Figure S1. Development of *Mtabi4* seeds
Figure S2. Germination speed of after-ripened seeds that were imbibed in the light or darkness.
Figure S3. Controlled deterioration test of *Mtabi4* seeds.
Figure S4. Tetrazolium staining Mtabi4 seeds shows loss of viability after ageing.
Figure S5. Longevity and chlorophyll content of Arabidopsis *abi4* seeds
Figure S6. Changes in transcript levels of genes involved in Chl degradation during maturation of *Mtabi5* seeds
Figure S7. Remanence of Chl in *Mtsgr* mutant seeds is associated with PSII and not linked to seed longevity.
Figure S8. Soluble sugar contents in dry mature seeds
Figure S9. Changes in transcript levels of *MtGUN1* and *MtGLK1* genes during maturation
Figure S10. Difference in the 2nd derivative spectra between 24- and 32-d old seeds.
Figure S11. Absence of photosynthetic electron transport during imbibition of *Mtabi4* seeds.

Supporting Tables:

Table S1. RNAseq dataset of *Mtabi4* seeds during seed maturation
Table S2. GO analysis of common transcripts between *Mtabi4* and *Mtabi5*
Table S3. Transcriptome comparison of developing Mtabi4 seeds with genes expressed in GLK1 sur-expressors in Arabidopsis leaves
Table S4 Oligonucleotide sequences of primers used in this study

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FIGURE LEGENDS

Figure 1. MtABI4 is orthologous to AtABI4 and regulates germination and seedling growth

a) Gene structure with *Tnt1* insertions and predicted APETELA 2 (AP2) domain, AP2-associated (AP2a) and LRP motifs.
b) Effect of ABA on germination. Data are the mean (± SE) of 3 replicates of 30 seeds. Seeds were incubated in the dark at 20°C and germination was scored after 7 days. Different letters between groups indicate significant difference using a Kruskall and Wallis test ($p<0.05$).
c) Germination percentage of indicated genotypes of Arabidopsis seeds in the presence of 2 µM ABA in the dark. *abi4* mutants were complemented with AtABI4 as a control and with MtABI4 under the Arabidopsis *ABI4* promoter. pABI4 (*abi4* seeds with an empty plasmid used as control). Data are the mean (± SE) of 3 replicates of *ca.* 100 seeds.
d) Mean germination time of freshly harvested seeds *Mtabi4* seeds imbibed at 20°C in the dark or continuous white light. Data are the mean (± SE) of 3 replicates of 30 seeds. Different letters between groups indicate significant difference using ANOVA and Tukey test ($p<0.05$).
e) Hypocotyl lengths measured on independent triplicates of 10 individuals after 6 days following transfer to light of seedlings that had germinated in the dark. Whiskers and symbols indicate respectively the 90th and 10th percentiles and outliers. Different letters between groups indicate significant difference using ANOVA and Tukey test ($p<0.05$).

Figure 2. *Mtabi4* seeds exhibit decreased longevity and chlorophyll retention

a) Changes in germination % during storage at 75% RH, 35°C of the indicated genotypes. Data represent the average (± SE) of 3 replicates of 30 seeds. assoc-WT refers to associated WT and corresponds to wild-type control plants that were simultaneously selected based on the absence of the *Tnt1* insertion in the MtABI4 gene
b) Representative picture showing the green seed phenotype during maturation of *Mabi4* seeds.
(c, d) Chlorophyll content at 32 DAP (c) and in mature seeds (d). Data are the means (± SE) of 3 replicates of 80-100 seeds. Stars indicate significant difference ($P<0.001$) using a t-test.
(e) Expression profiling of genes involved in chlorophyll biosynthesis (green column) and degradation (yellow column). Data are extracted from the RNAseq dataset (Table S1) at different developmental stages, 13 DAP, 24 DAP and 44 DAP (mature seeds). Stars indicate significant difference in transcript levels (adjusted p-values < 0.05 that was calculated using the Benjamini–Hochberg procedure to control the false discovery rate).
(f-h) Transcript levels of *MtGUN4* (f), *MtGUN5* (g) and *MtBCM1* (h) during seed maturation. Data are extracted from the RNAseq dataset (Table S1) and represent the mean of two biological replicates of 50 seeds from WT and *Mtabi4-1* for 13 and 24 DAP and three replicates of mature seeds of both *Mtabi4-1* and *Mtabi4-2*. Stars indicate significant difference in transcript levels (adjusted p-values < 0.05 that was calculated using the Benjamini–Hochberg procedure to control the false discovery rate).

Figure 3. MtABI4 and MtABI5 exert additive effects on longevity and degreening

a) Venn diagram comparing transcriptomes of *Mtabi4* and *Mtabi5* developing seeds. Transcriptome of *Mtabi5* seeds are from sZinsmeister *et al.* (2016)

b) Loss of viability during storage at 75% RH 35°C of indicated genotypes. Data are the means (± SE) of triplicates of 30 seeds.

(c-d) Chlorophyll contents of dry mature seeds (c) and representative image of the green seed phenotype of the indicated genotypes (d).

(e-h) Expression levels of genes involved in PSII (*MtLHCB2.1 (e), MtPSBW (f)*) and PSI (*MtLHCA1 (g), MtPsaD2 (h)*) complex in dry mature seeds. Data are the mean (± SE) of three biological replicates of 30 seeds. Different letters between groups indicate significant difference using ANOVA and Tukey test ($p<0.05$).

(i-k) Expression levels of genes involved in seed longevity (*MtEM1*, a LEA protein and *MtSIP1*, a raffinose synthase (j)) and *MtABI3* (k). Data are the mean (± SE) of three biological replicates of 30 seeds. Letters indicate significant differences ($p<0.05$) using a Kruskal-Wallis test (i) and ANOVA and Tukey test (j, k).

l) Expression levels of *MtABI4* in the indicated genotypes. Different letters between groups indicate significant difference using ANOVA and Tukey test ($p<0.05$).

m-n) *MtABI4* (m) and *MtEM1* (n) expression in *M. truncatula* leaves ectopically over-expressing *35S::MtABI5-GUS* in the presence of 0.5 µM ABA. Data are the mean (± SE) of technical duplicates of 4 independent transformants. Whiskers and symbols indicate respectively the 90th and 10th percentiles and outliers. Two-tailed t-test was used to calculate significance with plants over-expressing 35S::*GUS*.

Figure 4. *Mtabi4* seeds exhibit deregulation of genes associated with photosynthesis

a) Number of differentially expressed genes during maturation of *Mtabi4* seeds

b-c) Changes in *MtLHCB2.1* (MtrunA17_Chr5g0447531, b) and *MtLHCA1* (MtrunA17_Chr6g0462031, c) transcript levels as representative of photosynthesis associated nuclear encoded genes (PhANGs) from photosystem II and I (PS), respectively. Data were extracted from the RNAseq dataset (Table S1). Stars indicate significant difference in transcript levels (adjusted p-values < 0.05 that was calculated using the Benjamini–Hochberg procedure to control the false discovery rate).

d) GO enrichment analysis of the transcriptome of *Mtabi4* seeds during seed development. Green text indicates GO associated with plastid and photosynthesis. Italic text indicates cellular location.

Figure 5. *Mtabi4* seeds exhibit defective eoplast formation

a) Ratio between transcript levels of photosynthesis associated plastid encoded genes (PhAPGs) and photosynthesis associated nuclear encoded genes (PhANGs) in 24 d old and mature seeds. Genes were classified as associated with photosystem II (PSI) and I (PSI) and electron transport chain (ETC). Means of transcript levels of each class (4-17 genes, Table S1) were calculated to obtain the corresponding PhAPGs/PhANGs ratio.

b) Over-representation analysis of up-regulated transcripts in *Mtabi4* seeds harvested at 24 DAP and in mature seeds that were found in the list of co-regulated genes upon retrograde signalling (RS) activation during chloroplast biogenesis in Arabidopsis (data were obtained from (Martín *et al.*, 2016). Statistical over-representation was calculated using hypergeometric probability ($P<0.001$).

c) Representative micrographs of plastids of cotyledons from 32 DAP and mature seeds. Pictures were taken in the upper palisade layer. For the sake of comparison with dry mature seeds, 32-d old seeds were first dried prior to fixation. Both dried 32 DAP and mature cotyledons were first re-imbibed for 6h in the dark to allow penetration of glutaraldehyde. The arrow indicates the prolamelar body in the WT etioplast. Bars represent 0.2µm.

Figure 6. Dismantlement of light harvesting complex is perturbed in developing *Mtabi*4 seeds

a-e) Representative 77K fluorescence emission spectra of 24 d (a, b) and 32 d-old (c, d) and mature (e) seeds of the indicated genotype. Data were normalized to PSI peak value of the WT seeds. Panel b and d show the 2nd derivative of spectra of the corresponding left panel.

f) Calculated difference between the 77K fluorescence emission spectra taken before and after a flash of light applied after warming the sample at -20°C. The grey dashed line represents the results obtained with WT seeds.

Figure 7. Dysfunctional photosynthesis in *Mtabi4* seeds during maturation

a) Viability after indicated storage periods at 35°C, 75% RH of seeds that were obtained under control light conditions or from siliques that were covered with a black cloth throughout seed maturation. Data represent the average (± SE) of 3 replicates of 20-30 seeds. Star indicates significant difference at p=0.05 using a t-test. NS not significant.

b) Representative pictures of seeds of indicated genotypes harvested from pods that were grown under normal light and in darkness.

(c-e) Photochemical quenching analysis showing Fv/Fm (c), Sm (d) and DF (e) values. Data are the average of three independent biological replicates of 4-5 seeds. Different letters between groups indicate significant difference using ANOVA and Tukey test ($p<0.05$).

f) Over-representation of up-regulated transcripts in *Mtabi4* seeds from clusters characterizing transcriptional footprints provoked by reactive oxygen species (ROS) originating from GUN retrograde signalling (GUN retro), high light (HL), perturbing ETC in mitochondria in cell culture (ROS-CC), ROS incubation (ROS), $^1O_2$ and UVB light ($^1O_2$-UV) and in defective *rbohf* mutants. ROS clusters were from (Willems *et al.*, 2016). Statistical over-representation was calculated using hypergeometric probability ($P<0.001$).

Figure 8. Model summarizing the different actions of the ABA signaling pathway on seed longevity and eoplast formation

At the end of seed filling, MtABI4 initiates the dismantling of the light harvesting complexes which is accompanied by a decreased in Chl content. MtABI4 is at least partially under the regulatory control of *MtABI5*. In parallel, MtABI5 initiates the expression of genes associated with the synthesis of protective molecules that will ensure survival during storage and represses PHANGs expression (data from Zinsmeister *et al.*, 2016). The synergistic action of MtABI5 and MtABI4 ensures a coordinated shut-down on photosynthesis, thereby preventing oxidative stress during seed desiccation and/or in mature dry seeds. MtABI3 regulates degreening via activation of *MtSGR* (Lalanne *et al.*, 2021), a key gene involved in the early steps of Chl degradation and LHC dismantling. LEA, Late embryogenesis abundant; LHC, Light harvesting complex; PhANGs, Photosynthesis associated nuclear genes; SIP, Seed Imbition Protein (raffinose synthase). Arrows and bars indicate stimulation and inhibition, respectively. The dashed arrow indicates partial transcriptional control.