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The seed-specific heat shock factor A9 regulates the depth of dormancy in *Medicago truncatula* seeds via ABA signaling

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Abstract

During the later stages of seed maturation, two key adaptive traits are acquired that contribute to seed lifespan and dispersal, longevity and dormancy. The seed-specific heat shock transcription factor A9 is an important hub gene in the transcriptional network of late seed maturation. Here we demonstrate that HSFA9 plays a role in thermotolerance rather than in ex situ seed conservation. Storage of *hsfa9* seeds of *Medicago truncatula* and Arabidopsis had comparable lifespan at moderate storage relative humidity (RH), whereas at high RH, *hsfa9* seeds lost their viability much faster than wild type seeds. Furthermore, we show that in *M. truncatula*, *Mthsfa9* seeds acquired more dormancy during late maturation than wild type. Transient expression of *MtHSFA9* in hairy roots and transcriptome analysis of *Mthsfa9* Tnt1 insertion mutants identified a deregulation of genes involved in ABA biosynthesis, catabolism and signalling. Consistent with these results, *Mthsfa9* seeds exhibited increased ABA levels and higher sensitivity to ABA. These data suggest that in legumes, HSFA9 acts as a negative regulator of the depth of seed dormancy during seed development via the modulation of hormonal balance.

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INTRODUCTION

During development, seeds acquire a set of physiological characteristics that are essential to ensure the dispersion of the species and are therefore crucial for the establishment of seedlings in the field. These characteristics include dormancy and seed longevity, which play complementary roles in maintaining the embryo in a protected state in anticipation of favorable conditions to ensure germination (Finch-Savage & Bassel 2015; Penfield 2017; Leprince, Pellizzaro, Berriri & Buitink 2017). Both traits vary markedly among plant species with important consequences on plant phenology, establishment in the field, yield and *ex situ* conservation of the genetic diversity. Dormancy is an adaptive trait that inhibits freshly matured seeds to germinate under otherwise favorable conditions or out of the appropriate season. During storage and/or dispersal, mature seeds progressively lose dormancy over time, a process known as after-ripening. Longevity is defined as the ability to survive for extended periods of time during dry storage for seed crops and in soil seed banks for wild species (reviewed in Long *et al.* 2015; Sano *et al.* 2016; Leprince *et al.* 2016). Longevity depends on the ability of a seed to undergo complete desiccation without loss of viability. Dormancy and longevity are acquired progressively during seed maturation and are under the control of the maternal environment and mainly abscisic acid (ABA) (Graeber, Nakabayashi, Miatton, Leubner-Metzger & Soppe 2012; Zinsmeister *et al.* 2016; Penfield & MacGregor 2017).

In legumes, the acquisition of dormancy and longevity occurs during late seed maturation concomitantly with the upregulation of heat shock proteins (HSP) and small HSPs (sHSP) (Verdier *et al.* 2013; Lima*et al.* 2017). The expression of *HSP17.4* during seed maturation in Arabidopsis parallels the acquisition of dormancy and desiccation tolerance, and desiccation-intolerant mutants have decreased*HSP17.4* levels (Wehmeyer, Hernandez, Finkelstein & Vierling 1996; Wehmeyer & Vierling 2000). Several functional studies point to a role of sHSP in seed stress tolerance. In cabbage, the amount of HSP17.6 in dry seeds was positively correlated with germination under water stress conditions and after storage at 10% moisture and 42°C (Bettey & Finch-Savage 1998). Arabidopsis and rice seeds overexpressing*OsHSP18.2* , a sHSP that accumulates during late maturation, display an improved tolerance to controlled deterioration (CD) when stored at 100% RH and 45°C and to osmotic stress during germination (Kaur *et al.* 2015). sHSPs are thought to act as ATP-independent chaperones that bind stress-denaturing proteins to prevent their irreversible aggregation. The ATP-dependent chaperone machinery, composed of HSP70, HSP101 and other cochaperones can then proceed to refold sHSP-associated proteins (reviewed in (Ohama, Sato, Shinozaki & Yamaguchi-Shinozaki 2017; Jacob, Hirt & Bendahmane 2017).

Accumulation of HSPs is under the transcriptional control of heat shock factors (HSF), a large family of transcriptional factors represented by highly conserved structural features such as a N-terminal DNA binding domain (DBD) and an oligomerization domain composed of two hydrophobic heptad repeats (HR-A and HR-B) connected to DBD. Clade A of HSFs are characterized by the exclusive presence of AHA transactivator motif in their C-terminal trans-activation domain. To exert their function in stress tolerance, HSFAs are activated by ABA and function together with a set of transcription factors such as DREB2A and additional co-chaperones (Kotak, Vierling, Baumlein & Von Koskull-Dorlng 2007; Huang, Niu, Yang & Jinn 2016; Jacob *et al.* 2017; Bulgakov, Wu & Jinn 2019). Functional diversification was established among different HSFA members (Chauhan, Khurana, Agarwal & Khurana 2011; Jacob *et al.* 2017; Bulgakov *et al.* 2019). HSFA9 represents a unique member of the HSFA family that is specifically expressed in seeds during development and under the regulatory control of ABA INSENSITIVE 3 (ABI3) without the need of a heat shock (Kotak *et al.* 2007). Seed-specific overexpression of the *HSFA9* from *Helianthus annuum* in tobacco activated the expression of various HSPs (HSP101, sHSP-CI, sHSP-CII, and plastid sHSP) and transgenic seeds exhibited increased resistance to CD (Prieto-Dapena, Castano, Almoguera & Jordano 2006). In contrast, *HaHSFA9* repression in tobacco seeds using an active repressor version *HSFA9-SRDX* resulted in the reduction of seed-specific sHSP proteins and a decreased tolerance against CD but did not affect desiccation tolerance (Tejedor-Cano *et al.* 2010), suggesting a specific role in seed longevity. Additional phenotypes observed in *HaHSFA9* overexpression lines are related to the protection of the photosynthesis apparatus from dehydration and oxidative stress in tobacco seedlings (Almoguera *et al.* 2012) or tolerance to severe water loss (Prieto-Dapena, Castano, Almoguera & Jordano 2008).

In *Medicago truncatula,* the construction of a gene regulatory network of transcription factors preferentially expressed in seeds identified a homologue of *HSFA9* as the connecting node between longevity and desiccation tolerance modules (Verdier *et al.*2013). The objective of this work was to investigate the role of HSFA9 in the regulation of seed longevity and vigor in *M. truncatula* . In the dry state, seeds can survive for many years, making it difficult to assess their longevity within a reasonable time (Hay, Valdez, Lee, Sta Cruz & Sta. Cruz 2018). To accelerate the deterioration of seeds during storage in a tractable time scale, CD or accelerating ageing tests have been proposed by increasing the RH at or above 85% and temperatures over 40degC during storage, conditions used to assess the role of HSFA9 in previous studies. However, there are increasing concerns from biophysics and genetics studies about the reliability of these tests as a proxy to evaluate seed longevity *ex situ* in conditions typically found in gene banks where the low RH and temperature allowed the seeds to enter into a solid-like state as opposed to a fluid state (reviewed in (Leprince *et al.* 2017; Hay*et al.* 2018; Ballesteros & Walters 2019; Zinsmeister, Leprince & Buitink 2020). Here we discovered that seed survival was only affected in *hsfa9* mutants from *M. truncatula* and Arabidopsis when water contents in the seeds exceeded approx. 0.2 g H_2O g DW, showing that HSFA9 has a role in thermotolerance during wet storage, when the cytoplasm is in a fluid state. Our study also reveals an unexpected role for MtHSFA9 in the regulation of the depth of seed dormancy in *M. truncatula* . Further analysis of putative targets

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indicated a strong deregulation of genes involved in ABA and gibberellin (GA) metabolism and signaling, translated in an increase in ABA content and hypersensitivity to ABA in the Mt*hsfa9* seeds.

Experimental procedures

Plant material and growth conditions

Three independent Tnt1 insertion lines in the *HSFA* gene Medtr4g126070 of *M. truncatula,* Mt*hsfa9-1* (NF10440), 6 bp from ATG), Mt*hsfa9-2* (NF13157, *4* 6 bp from ATG) and Mt*hsfa9-3* (NF12877,1333 bp from ATG) in the R108 background were obtained after screening by the Noble Foundation. Homozygous lines were screened by PCR with the primers listed in Table S1. For each allele, wild type control plants were simultaneously selected based on the absence of the Tnt1 insertion in the *MtHSFA9* gene and are referred to as associated wild type. The homozygous Mt*hsfa9-1*line was backcrossed once with the pollen of the wild type R108. Arabidopsis insertion lines in AtHSFA9 (At5g54070) were obtained from ABRC. Among the three T-DNA insertion lines ordered, homozygous lines were only identified for the Salk_062453 using primers in Table S1.

M. truncatula plants from the different genotypes were grown in batched for 10 plants trays containing a sterile mix of vermiculite at 20degC/19degC, with a 16 h light photoperiod at 200 µmol photons m -**²**s-**²**. For the high temperature treatment during seed development, plants were transferred to a growth room at 26°C with similar light conditions when 10 flowers had appeared. Flowers were tagged and developing seeds were harvested at different time intervals until pod abscission and after final desiccation. At the abscission stage the pods were dried for 3 days at a relative humidity of 44% generated by saturated solution of K_2CO_3 at 20°C. The seeds were manually removed from the pods and stored at 20°C in the dark for further after-ripening or kept at -20°C. Arabidopsis plants were grown at 20°C/18°C with a photoperiod of 16 hours. The seeds were harvested after pod abscission and then dried for three days at 44% RH, generated by saturated solution of K_2CO_3 at 20 °C.

Physiological tests

For all tests, three replicates of 30 seeds each for *M. truncatula* or three replicates of 150-200 seeds each of Arabidopsis were used unless mentioned otherwise. To test for germination, *M.truncatula* seeds were scarified with a sandpaper and imbibed in the dark at 20°C (Chatelain *et al.* 2012). Seeds of *A. thaliana* were imbibed at 20°C and a photoperiod of 16 hours light. For both species, germinated seeds were scored at regular time intervals by counting seeds having an emerged radicle with length [?] 1 mm. To release physiological dormancy prior to germination tests, scarified seeds of*M. truncatula* were imbibed at 20 degC for 4 h in Petri dishes onto filter papers then transferred at 4 degC for three days in the dark. For *A. thaliana,* seeds were imbibed for 3 days directly at 4degC in the dark. Water contents were assessed gravimetrically for triplicate samples of 5 seeds by determination of the fresh weight and subsequent dry weight after 2 d in an oven at 96degC. Water contents are expressed on a dry weight basis. Seed weight of the genotypes was measured on five replicates of 50 seeds after oven drying at 96degC for 2d.

For accelerated aging assays, scarified seeds of *M. truncatula* or mature seeds of Arabidopsis were placed over a saturated solution of NaCl (75% RH) at 35degC in hermetically sealed boxes and viability was evaluated by germination at regular time intervals. Longevity was expressed as P50, defined as the time (days) at which the stored seed lost 50% of viability during storage. Controlled deterioration assays were carried out by equilibration of seeds at 85% RH (saturated KCl) or incubation in water vapor (100%) and 35degC or 40degC.

The effect of ABA inhibition on the T50, the time of imbibition needed to reach 50% of germination, was determined by imbibition of mature seeds of *M. truncatula* in a 10 µM fluridone solution freshly prepared from a 1 mM stock solution where fluridone was dissolved in ethanol and Tween 20 solution (2/1 v/v).

ABA sensitivity was determined by imbibition of mature, 4 months after ripened seeds on filter paper on a range of ABA concentrations (mixed isomers, Sigma, St Louis, MO, USA) at 20°C, 16h light or in the dark. ABA was dissolved in methanol prior to dilution in water. Control seeds were imbibed in the MeOH

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concentration corresponding to the highest ABA concentration (0.05% MeOH). Germination was scored after 14 days.

To evaluate dormancy model of the Mt*hsfa9* mutants is imposed by the embryo or by the surrounding tissues (testa, endosperm) or combinations of these tissues, three replicates of 30 seeds were imbibed for 6 hours at 20 ° C. Using a magnifying glass, the endosperm and seed coat were carefully removed, taking care of not damaging the embryos. Scarified intact seeds and naked embryos were imbibed in water at 25 ° C in the dark. Germination (for intact seeds) or embryo growth were recorded over time.

Soluble sugar quantification

Three replicates of 30 seeds each were lyophilized and dry weight was determined gravimetrically. Sugars were extracted and analysed by HPLC on a Carbopac PA-1 column (Dionex Corp.) as described by Rosnoblet et al. (2007).

ABA quantification

ABA content was measured using the Phytodetek® Immunoassay Kit for ABA (Agdia) according to the manufacturer's instructions. Extraction was performed on 3 biological replicates of 25 mature seeds 1 month after harvest according to Jiang and Zhang (2001).

RNA extraction and Quantitative real time PCR

RNA extraction was performed on 3 replicates of 30 seeds each that were ground in liquid nitrogen using mortar and pestle. Total RNA was extracted using the NucleoSpin RNA Plant Kit (Macherey Nagel) according to the manufacturer instruction. cDNA synthesis was carried out with the iScript Ready-to-Use cDNA Supermix (Bio-Rad Laboratories, Inc) according to the manufacturer instructions. RT- qPCR was performed with SsoAdvanced Universal SYBR(r) Green Supermix (Bio-Rad Laboratories). Quantification of transcript levels were performed on a CFX96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) with primers shown in Table S1. Reference genes were *MtTCTP* and *ACTIN11* commonly used in the laboratory for *M. truncatula* qRT-PCR analysis (Zinsmeister et al., 2016). The relative expression (RE) was normalized with the geometric mean of the 2 reference genes previously mentioned and was calculated according to the following formula:$PE= 2^{\hat{}\Delta\tilde{}\tau}$ (where $\Delta\tilde{}\tau$ = geometric mean of the Ct reference genes - Ct target gene) with Ct is the value of the detection cycle of the transcript. Each point represents the average of three independent biological replicates of 30 seeds each.

RNAseq and data analysis

For RNAseq analysis, RNA was extracted from three replicates of 25 mature seeds of wild type, *Mthsfa9-1* and *Mthsfa9-2* alleles that were grown together. RNAseq was outsourced to BGI Genomics (Shenzhen, China). RNA quality was checked with an Agilent 2100 Bio analyzer (Agilent RNA 6000 Nano Kit). RNA was sequenced using the DNBseq platform, generating an average 24M reads per sample. The sequencing reads which containing low-quality, adaptor-polluted and high content of unknown base (N) reads were removed and mapping of the clean reads was performed against the Mt4.0 genome. Total mapping ratio was 90%, and unique mapping ratio 78%. DEGs (differential expressed genes) between samples were determined using DEseq2 algorithms. Genes were considered differential when the adjusted P-value was <0.05 and log2FoldChange of 1. On the DEGs, GO (http://www.geneontology.org/) and KEGG (https://www.kegg.jp/) enrichment analysis of annotated different expression gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on Hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value [?] 0.05) by Bonferroni. RNAseq data were deposited in the NCBI Gene Expression Omnibus database (accession number GSE98199 and GSExxxx).

Transcriptomic analysis of *Medicago truncatula* hairy roots overexpressing 35S::MtHSFA9::GFP compared to 35S::GUS::GFP

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For ectopic overexpression of *MtHSFA9* ,*35S::MtHSFA9::GFP/35::GUS* and the control (plasmid containing the *GUS* gene alone) plasmids were obtained by the gateway cloning method according to Verdier et al (2013) and used for*Agrobacterium rhizogenes* transformation for subsequent hairy root transformation in *Medicago truncatula* following the protocol of (Boisson-Dernier *et al.* 2001). *MtHSFA9* and *GUS*amplicons were obtained by PCR using primers listed in Table S1 and cloned in pK7GW2D,1 (Karimi, Inzé & Depicker 2002) containing a 35S cauliflower mosaic virus promoter and GFP reporter that was used as transformation marker. Seedlings were grown in square Petri dishes with Fahraeus medium two months at 20°C, 16h light, at an angle of 45°. Transformed roots were selected by GFP fluorescence under UV microscope (stereomicroscope, Olympus U-RFL-T) and harvested in liquid nitrogen. RNA was extracted from three replicates of similar amounts of roots using 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). Transcriptome data has been submitted to the Gene Expression Omnibus as GSExxxxx.

Phylogenetic analysis

The 14 HSFA amino acid sequences of Arabidopsis were retrieved from the TAIR data base. The amino sequence of MtHSFA9 was blasted against the legume transcription factor data base LegumeTFDB (*http://legumetfdb.psc.riken.jp/*) containing sequences from soybean, *M. truncatula* and *Lotus japonica* . The resulting gene list was manually curated to retain only orthologs of the HSFA family. Since the Medicago gene was originally annotated as HSA2.2, we added the amino sequence from two HSFA2 genes already characterized in the literature, TsHSFA2d and OsHSFA2d (Chauhan*et al.* 2011; Chauhan, Khurana, Agarwal, Khurana & Khurana 2013). This orthologous gene set together with HaHSFA9 was used to construct a phylogenetic tree using neighbour-joining algorithm from the MEGA7.0 software (Kumar, Stecher & Tamura 2016).

Yeast-One Hybrid

Fragments of 60 bp regions of promotors of *MtNCED* genes*Medtr5g025230, Medtr5g025250, Medtr5g02527,* predicted to contain a putative binding site for *MtHSFA9*(*http://plantpan2.itps.ncku.edu.tw*) (Table S1) were cloned into pBait-AbAi vector as bait constructs. The coding sequence of *MtHSFA9* was cloned into the pGADT7-Rec AD cloning vector as prey construct. The empty pGADT7-Rec plasmid was used as negative control. Transformation and screening process were carried out on the media containing SD-Leu and SD-Leu + AbA antibiotic, respectively, according to the manufacturers' instructions (*www.clontech.com*).

RESULTS

Identification of the seed-specific MtHSFA9 in *M. truncatula* as the ortholog of HaHSFA9

To identify transcription factors (TF) that underly the regulation of the acquisition of desiccation tolerance (DT) and longevity, we performed a detailed temporal transcriptome study during seed development (Verdier *et al.* 2013). The construction of a gene co-expression network of genes and seed specific transcription factors that correlated with DT and longevity inferred a number of TFs as putative regulators of late maturation/longevity genes (Verdier *et al.* 2013). Detailed characterization of this network identified a heat shock transcription factor (Medtr4g126070/HSFA) as a central hub of the module, with the highest betweenness centrality (Fig. 1a, Data S1). In addition, this HSFA was one of the nodes with the closest centrality and number of connected nodes, with only Medtr1g023170, a DREB2D TF, being more connected (Data S1).

A blast of the *M. truncatula* sequence against Arabidopsis TAIR11 revealed homology with three AtHSFA: At2g26150/HSFA2 (7.55e-75), At3g22830/HSFA6B (1.25e-72) and AT5G54070/AtHSFA9 (7.17e-72). To analyze to which group the *M. truncatula* HSFA belongs, a phylogenic tree was constructed using homologous HSFA protein sequences retrieved from*M. truncatula, Glycine max* and *Lotus japonica* databases (Fig. 1b). The phylogenetic reconstruction was performed using maximum parsimony and identified Medtr4g126070 as part of a subgroup containing AtHSFA9, HaHSFA9, GmHSFA9.1 and GmHSFA9.2 (Fig. 1b). We therefore

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referred to the gene as *MtHSFA9* . Since the Arabidopsis and sunflower HSFA9 are specifically expressed in seeds, we investigated the expression patterns of *MtHSA9* and the other *HSFA* genes (Fig. 1c). *MtHSFA9* is predominantly expressed in seed tissues, with the highest transcript levels at the end of seed maturation (36 days after pollination (DAP)) (Fig. 1c, Fig. S1a). A moderate level of transcript was also detected in shoots submitted to severe drought.

Identification of downstream targets of MtHSFA9

As a first proxy to understand the function of MtHSFA9, putative target genes were identified using ectopic expression in hairy roots of*M. truncatula* followed by transcriptome analysis. A total of 420 transcripts were significantly up-regulated, and 253 transcripts downregulated in the*35S::MtHSFA9::GFP* hairy roots compared to roots transformed with a control plasmid ($p<0.01$) (Fig. 2a). A total of 11 HSP and sHSP, and three HSF were amongst the highest differentially expressed genes (Data S2). Next we identified two knock out lines in the MtHSFA9 gene, Mthsfa9-1 and Mthsfa9-2 with TNT1 insertions at position 6 and 46 respectively from ATG (Fig. S1b). For each line an associated with type was also selected, corresponding to the line without an insertion at the MtHSFA9 genes at the corresponding position. RNA seq analysis was performed on Mthsfa9 mutants and identified 2323 down-regulated and 2408 up-regulated transcripts in mature mutant seeds compared to wild type seeds (Fig. 2a, Fig. S1c, Data S3). A Venn diagram shows the overlap between the data set from the hairy roots and deregulated genes in the mutant seeds compared to their respective controls (Fig. 2a, Dataset S2). A total of 95 genes were identified as putative targets of *MtHSFA9* , with 67 positively regulated and 28 negatively regulated. RT-qPCR validated the reduced transcript level in mature Mt*hsfa9* seeds for*MtHSP70* , *MtHSP18.2* and *MtHSP17.5* compared to the wild type and associated wild type seeds (Fig 2b-d). Several putative target genes positively regulated by MtHSFA9 encoded members of the class I and class II HSP family (5), three HSP70 and two additional HSF,*HSFB2A* and *HSFA2* . Projection of these putative targets on the co-expression gene network identified 10 genes, out of which eight were directly connected to *MtHSFA9* and one connected with Mt*HSFB1* , another HSF that was deregulated when *MtHSFA9* is mutated (Fig. S2a). Other putative targets positively regulated by MtHSFA9 and known to be involved in the regulation of the heat shock response were the co-chaperone regulator peptidyl-prolyl cis-trans isomerase *FKBP65/ROF1* (Meiri & Breiman 2009) and*BCL-2-associated athanogene6/BAG6* (Nishizawa-Yokoi, Yoshida, Yabuta & Shigeoka 2009). Also, genes encoding for enzymes involved in the synthesis of raffinose family oligosaccharide (RFO seed imbibition 2, a raffinose synthase and galactinol synthase). Analysis of the soluble sugar content in mature seeds of the *Mthsfa9* mutants revealed that glucose contents were higher compared to wild type and associated wild type seeds (Fig. 2e), whereas verbascose content was lower (Fig. 2h). No significant difference was detected for sucrose and stachyose, the major soluble sugars in mature seeds (Fig. 2f-g).

Further identification of the biological functions of the downstream pathways modified by MtHSFA9 was investigated by KEGG mapping and Gene Ontology (GO) enrichment (Fig. 2i and Fig. S2b). The main KEGG pathways that were significantly deregulated in the Mt*hsfa9* seeds were related to biosynthesis of secondary metabolites (flavonoid biosynthesis), carotenoid biosynthesis, porphyrin and chlorophyll metabolism, glutathione metabolism, sphingolipid metabolism, carbon fixation and carbon metabolism (Fig. 2e). Enriched GO categories that were complementary to the KEGG mapped pathways included transport, DNA replication and initiation, sulfur compounds and response to abiotic stimulus (containing all the HSP/HSF) (Fig. S2b).

HSFA9 is not implicated in seed longevity but improves thermotolerance during wet storage

The main function identified for the sunflower homologue of HSFA9 is a role in seed deterioration during storage (Prieto-Dapena *et al.* , 2006; Tejedor-Cano *et al.* , 2010). To investigate if MtHSFA9 is involved in seed longevity, seeds of wild type and Mt*hsfa9*mutants were produced under standard growth conditions, and viability loss was followed during storage using moderate accelerated storage conditions (75% RH and 35°C). No difference could be detected in longevity, both the survival curves and the P50, determined as the storage time needed for the seed population to lose 50% of their viability, were similar between wild types and Mt*hsfa9* mutants (Fig. 3a). Two additional cultures were grown and storage experiments were

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conducted on the harvested seeds, and this confirmed the absence of a perturbed longevity phenotype of the Mt*hsfa9* mutants (Fig. S3a,b).

Next, we hypothesized that MtHSFA9 might have a role in the regulation of longevity when the mother plants are grown under high temperature conditions. Indeed in *M. truncatula,* high temperature (26°C) was shown to reduce longevity in the A17 genotype (Righetti et al., 2015). To test this hypothesis, wild type and mutant seeds were produced at 26°C, which shortened seed development compared to 20°C for all genotypes, with pod abscission occurring at 552-575°days at 26°C compared to 731-748°days at 20°C. Seed filling was significantly impacted by the high temperature, in a similar manner for the Mt*hsfa9* mutants and wild type (Fig. S4a). Longevity was significantly higher for all genotypes when seeds were produced at 26°C compared to 20°C (Fig. S4b and c). However, no significant differences were observed between wild type and Mt*hsfa9* seeds. We then examined if high temperature affected transcript levels of putative target genes of MtHSFA9 (Fig. S4d-f). Whereas *HSP70* transcript levels were lower in wild type seeds grown at 26°C compared to 20°C,*MtHSP17.5* and *MtHSP18* .2 transcripts significantly increased during the high temperature treatment (Fig. S4e-f). Yet, transcript levels of these three genes were not significantly affected by heat in the Mt*hsfa9* mutants compared to the wild type seeds.

Since no longevity phenotype could be observed in the Mt*hsfa9*seeds, we hypothesized that the difference between our study and previous works (Prieto-Dapena *et al.* , 2006; Tejedor-Cano *et al.* , 2010) might originate from differences in the seed aging protocols. To verify this, we used the Controlled Deterioration Test (CDT) protocol by equilibration at 100% RH and 40°C, that we refer to as 'wet aging'. Under these conditions, seeds of the Mt*hsfa9*mutants lost their viability much faster than wild type seeds, showing over three-fold reduction in lifespan compared to wild type seeds (Fig. 3b). We further investigated the effect of RH on seed lots that were after-ripened for 7 months and submitted to the same storage temperature (35°C) but different RH (Fig. S5a). Storage of seeds at 100% RH/35°C or 85% RH/35°C also showed a faster deterioration phenotype for the Mt*hsfa9* mutants, evident from a reduction in germination speed and final germination percentage (Fig. S5b,c). Water contents at the different storage conditions after 56 days of equilibration were 0.14 g H_2O/g DW at 75% RH, 0.18 g H_2O/g DW at 85% RH and 0.84 g H_2O/g DW at 100% RH regardless of the genotype (Fig. S5d). This indicated that elevating the water contents to allow metabolism activates protective or repair processes governed by HSFA9 that do not occur during dry aging.

To verify whether these findings were only applicable to HSFA9 from*M. truncatula* , we isolated an Arabidopsis T-DNA insertion mutant in which transcript level of *HSFA9* (At5g54070*)* was strongly reduced (Fig. S6a,b). The transcripts of two putative targets,*HSP17.4* and *HSP17.6* (Almoguera *et al.* 2002; Kotak*et al.* 2007) were significantly decreased in mature mutant seeds, thereby demonstrating that HSFA9 is a transcriptional activator of these two HSPs in Arabidopsis seeds (Fig. S6c). Like in *M. truncatula* , mature seeds of the wild type and At*hsfa9* mutants lost their viability at a comparable rate during storage at 75% RH and 35°C (Fig. 3c). In contrast, when seeds were submitted to the CDT protocol, At*hsfa9* mutants lost their viability much faster than wild type (Fig. 3d). This suggests that HSFA9 or downstream targets begin to function in the seeds when water content increases through equilibration between 75% and 85% RH.

MtHSFA9 plays a role in the release of dormancy during late maturation

When the viability of the seed lots was tested, we noticed that promptly after harvest, seeds took a very long time to germinate. Since *M. truncatula* seeds exhibit physiological dormancy, apparent from a strong reduction in germination speed that is released during after-ripening (Bolingue et al, 2010), we decided to further investigate this behaviour. Seeds of the different genotypes were harvested at pod abscission and dried for three days at 43% RH to homogenize the water content before imbibition. Whereas wild type seeds germinated within 2-3 days, seeds from Mt*hsfa9* lines germinated at around 8-10 days (Fig. 4a). Stratification for 3d at 4°C drastically increased the germination speed of the mutants, and only a very slight difference was still apparent between wild type and Mt*hsfa9* lines (Fig. 4b). During after-ripening, the difference in germination speed between the mutants and wild type seeds remained significant up until 5 months, but the relative rate of after-ripening did not differ between the genotypes (Fig. 4c).

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Since Mt*HSFA9* transcript levels increased around 20 DAP and reached a maximum at 36 DAP (Fig. S1a), dormancy levels were determined as mean germination time in freshly harvested seeds from 20 DAP until pod abscission (Fig. 4d). At 20 DAP, prior to the acquisition of dormancy (Bolingue, Vu, Leprince & Buitink 2010), no difference was found in the dormancy levels between the genotypes (Fig. S7a). Significant differences were observed at final maturation, with the highest difference prior to full maturation drying, at 40 DAP (Fig. 4d, Fig. S7b). Absence of MtHSFA9 doubled the mean germination time from 35 to 70d, indicating that MtHSFA9 regulates the depth of dormancy acquisition during late maturation. Seed development at 26°C did not change the dormancy level in the Mt*hsfa9* mutants compared to wild type (Fig. S7c). Freshly harvested Arabidopsis *hsfa9* seeds showed reduced germination but this was comparable to wild type seeds (Fig. S7d). After-ripening increased the germination percentage and speed, but no difference could be detected between the At*hsfa9* and wild type seeds (Fig. S7d).

HSFA9 regulates dormancy via modulation of ABA metabolism and signaling

To understand the increased dormancy phenotype of the Mt*hsfa9*seeds, we searched for evidence whether ABA or GA signaling pathways were affected by comparing the transcriptomes of *Mthsfa9* seeds and hairy roots ectopically expressing *MtHSFA9* . For ABA, five genes were differentially expressed in both analyses. Three genes had higher transcript levels in the Mt*hsfa9* mutants, and were downregulated when *MtHSFA9* was expressed in the hairy roots: an*indole-3-acetaldehyde oxidase* (*AAO* , Medtr5g087410) that is involved in ABA biosynthesis, *PYL/PYR6* (Medtr5g083270), an ABA receptor, and *HVA22* , a marker gene of ABA response (Data S2 and Fig. 5a). In addition, a cytochrome P450 (*CYP707A* Medtr8g072260), involved in ABA degradation, was also differentially expressed in both studies. We also noticed that one of the most significantly overrepresented GO/KEGG category in the Mt*hsfa9* mutants was carotenoid metabolism (Fig. 2i, Fig S2b). A closer look at the genes in this pathway revealed that those involved in ABA biosynthesis were upregulated, including a zeaxanthin epoxidase (*ZEP)* and three 9-cis-epoxycarotenoid dioxygenase *(NCED)* , whereas two other*CYP707A* genes were downregulated (Fig. 5a), suggesting that MtHSFA9 could regulate ABA homeostasis. RT-qPCR confirmed the differential expression of these genes in mature seeds from wild type and Mt*hsfa9* mutants from a different harvest (Fig. 5b-d). For GA, the comparison of both transcriptomes revealed the biosynthesis gene gibberellin 3-beta- dioxygenase 1 (*GA3OX1)* being downregulated in the Mt*hsfa9* seeds and upregulated in the transgenic hairy roots. In addition, several other genes related to GA metabolism were deregulated in the Mt*hsfa9* seeds, namely two orthologs of gibberellin 2-beta-dioxygenase 2 (*GA2OX2)* involved in GA inactivation that were upregulated in the mutants, and one gene involved in GA biosynthesis, gibberellin 20 oxidase 2 (*GA20OX2)* , that was downregulated. A GA receptor, *GID1* was also found to have reduced transcript levels in the Mt*hsfa9* seeds. Another putative link between GA is suggested by the deregulation of the homolog of the protein *REVEILLE1* (*RVE1*) both in hairy roots and Mt*hsfa9* seeds (Data S2). This gene was shown to directly inhibits*GA3OX2* transcription in Arabidopsis and affect dormancy (Yang, Jiang, Liu & Lin 2020). All these results are consistent with the dormancy phenotype in *Mthsfa9* seeds.

Considering the higher transcript levels of ABA biosynthesis genes and decreased levels of ABA degradation genes, ABA content was determined in mature seeds. A significant increase in ABA was observed in the mature Mt*hsfa9* seeds compared to the wild type seeds, with an increase from 317 to 511 and 576 fmol/g DW for Mt*hsfa9-1* and Mt*hsfa9-2,* respectively (Fig. 6a). To investigate whether the dormancy phenotype of Mt*hsfa9* seeds was due to further ABA biosynthesis during imbibition, freshly harvested seeds were imbibed in the ABA inhibitor fluridone. For all genotypes, fluridone promoted germination speed. However, compared to their respective mock controls, the impact of the fluridone treatment on the time to 50% germination (T50) was not significantly different between Mt*hsfa9* and wild type seeds (Fig. 6b). This suggests that dormancy in *Mthsfa9*seeds could not be explained by *de novo* ABA synthesis during imbibition but rather originates from differences induced during seed maturation. Further evidence that the dormancy phenotype of Mt*hsfa9* is related to the maturation program and not activated during imbibition comes from the expression of the three hormone metabolism genes that were deregulated in the Mt*hsfa9* mutants:*MtNCED4* , *MtCYP707A* and *MtGA3OX1* . These three genes showed clear differential expression profiles during imbibition between dormant and non-dormant wild type seeds (Fig. S9a-c). However, imbibition of Mt*hsfa9* seeds did not show

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this typical dormancy profile (Fig. S9d-f).

Besides differences in ABA metabolism, several other ABA signaling genes were deregulated in the Mt*hsfa9* mutants (ABA receptor*PYL6* , protein phosphatase 2C, *PP2C* , BURP domain protein*RD22* , *HVA22 protein;* Fig. 5a) suggesting that*Mthsfa9* seeds could exhibit altered ABA sensitivity during imbibition. This was tested by incubating after-ripened seeds in increasing ABA concentrations (Fig. 6c). Mutant seeds showed hypersensitivity when imbibed in darkness compared to wild type (Fig. 6c).

Physiological dormancy can be imposed by the embryo or the surrounding tissues (testa, endosperm) or combinations of these tissues, and their sum and interaction determine the depth of dormancy (Chahtane, Kim & Lopez-Molina 2016; Penfield 2017). To evaluate whether the dormancy phenotype of the Mt*hsfa9* mutants came from the embryo or the covering structures, isolated embryos were incubated in water at 25°C and radicle growth was monitored (Fig. 6d). For all genotypes, embryo radicles grew faster in coat-less seeds, as previously observed (Bolingue *et al.* 2010). However, the time for the radicle to initiate growth was longer for the isolated *Mthsfa9* embryos compared to the wild type embryos and the final percentage of seeds with radicles that had grown was smaller in *Mthsfa9* compared to wild type (Fig. 6d, Fig. S7e). Thus, the difference in germination/growth in the mutants remains visible without the surrounding layers and appears to reside mostly in the embryo.

The heat shock elements (HSE) that HSFA9 binds to has been shown to bind a modified HSE (Carranco, Almoguera & Jordano 1999). It was suggested that the specific sequence of this heat stress cis-element is crucial for HsfA9 promoter selectivity, and that this selectivity could involve preferential transcriptional activation following DNA binding. We investigated if a similar pattern could be observed for the promotors of the putative MtHSFA9 targets, starting with the two most likely targets: HSP17.5 and MtHSP18.2 (Fig. 2b, c; Kotak et al., 2007). The promotor of HSP17.5 contained one perfect HSE (Table S2, Guo *et al.* , 2008). HSP18.2 did not contain any perfect HSE, and neither of the two promotors contained the modified HSE detected for HaHSAF9. For the other 22 hormone-related genes that were deregulated in the Mt*hsfa9*mutants (Fig. 5; Table S2), all HSE sites contained at least 2 out of 9 mismatches, but no common sequence could be found that could be tested as a putative binding site for MtHSFA9. However, for six genes (*MtPYL6, MtPP2C, AAO, NCED, HVA22* and *Ga20Ox*), a similar modified sequence GAAnnTTXnnXAA was identified, and three of these genes also show deregulation in the hairy root ectopically expressing*MtHSFA9* (Fig. 5). Since *MtNCED4* was one of the most differentially expressed genes (Fig. 5a), a yeast one hybrid assay was carried out to determine if MtHSFA9 could bind to the putative HSE present in the promoter region of this gene (Fig. S8). No interaction could be detected, suggesting that this gene is either not under the direct regulation of MtHSFA9 or that other parts of the promotor are needed for the interaction.

DISCUSSION

HSFA gene family members play a crucial role in plant response to several abiotic stresses by regulating the expression of stress-responsive genes including heat shock proteins (Ohama *et al.* 2017; Jacob *et al.* 2017). A peculiar HSF, HSFA9, has been reported in several species with an expression being restricted to seeds, more precisely increasing during the final part of seed development (Almoguera *et al.* 2002; Kotak *et al.* 2007; Li*et al.* 2015). This work tested whether a putative homologue of HSFA9 from *M. truncatula,* a hub gene involved in seed maturation (Fig. 1) plays a role in seed longevity, defined as the time seeds remain viable after dry storage. We demonstrated that at mild storage conditions, *hsfa9* mutants of both *M. truncatula* and Arabidopsis did not show an altered longevity phenotype, but more humid and hot storage conditions revealed a strong decrease in seed viability (Fig. 3), the later corroborating with earlier studies using heterologous ectopic expression in tobacco (Prieto-Dapena *et al.* , 2006; Tejedor-Cano *et al.* , 2010). Further physiological characterization of Mt*hsfa9* mutant seeds combined with transcriptomic analysis revealed that *MtHSFA9* is a regulator of the depth of seed dormancy during seed maturation via the modulation of ABA homeostasis and signalling.

Our data show that HSFA9 does not play a role in seed longevity when mild storage conditions are used. Only when moisture in the seeds was increased by the CD test, seed viability was lost at a faster rate in the

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hsfa9 mutants. It is well known that the water content and temperature combination in the seeds during storage will determine the rate and type of ageing reactions (Buitink & Leprince 2008; Ballesteros & Walters 2019; Zinsmeister *et al.* 2020). In our system, seeds were exposed to mild storage conditions (75% RH, 35°C) whereas transgenic tobacco seeds over-expressing *HaHSFA9* were exposed to 42°C and 100% RH (Prieto-Dapena *et al.* 2008). Under these conditions, seeds imbibe and resume metabolism while tolerating the heat stress, considering that the onset of respiration is at 90-92 % RH for a wide range of species (Vertucci & Roos 1990). Consistent with this,*OsHSP18.2* gene expression increased 60 fold when rice seeds were stored for 6 days at 45°C, 100% RH (Kaur *et al.* 2015). These storage conditions are far from ours where the seed water content was still low enough to prevent resumption of metabolism during storage. There are increasing concerns with regard to the similarity between accelerated aging conditions such as the CD test and so called natural/ambient aging conditions (Schwember & Bradford 2010; Nagel*et al.* 2015; Zinsmeister *et al.* 2016; Roach, Nagel, Börner, Eberle & Kranner 2018; Hay *et al.* 2018). Here we demonstrate that at least for the *hsfa9* phenotype, results obtained by these methods are not comparable and it is evident that care has to be taken in extrapolating accelerated aging conditions to dry storage. Hay and colleagues (Hay *et al.* 2018) recommended that seed ageing protocols should be designed based on the potential downstream use of the findings and the biological significance of longevity. Therefore, the role of HSFA9 might be confined to situations in nature, when seeds are buried in the soil and submitted to rehydration-dehydration cycles rather than situations where seeds are stored in the dry state for gene banking purposes. It would be interesting to know if under more moderate soil temperatures (10-25°C), HSFA9 still exerts its protective function on seed viability. Some suggestion that this might be the case comes from the increased tolerance against osmotic stress during imbibition in tobacco seedlings over-expressing HaHSFA9 (Prietro-Dapena et al., 2008) and in Arabidopsis seeds over-expressing a sHSP of rice (Kaur *et al.* 2015). The most plausible downstream genes regulated by HSFA9 that confer the protection during wet and hot storage are likely to be HSP, since their modulation correlates with seed viability (Prieto-Dapena *et al.* 2006; Tejedor-Cano *et al.* 2010; Kaur *et al.* 2015). Our transcriptome data is consistent with this as MtHSFA9 modulated the expression several (s)HSP (Data S2). Considering the chaperone function of sHSP, the absence of decreased longevity in *hsfa9* seeds raises the intriguing possibility that protein aggregation or misfolding might not occur in the dry state where molecular motion is strongly constrained (Buitink 2000, Ballesteros and Walters 2019). Besides sHSP, our transcriptomic data revealed that several components of the chaperone signalling involved in thermotolerance are being deregulated, namely three HSP70, two additional HSF, HSFB2A and HSFA2, as well as ROF1 and BAG6. Interestingly, HSFA2 is a direct target of the master regulator HSFA1 and essential in the induction of the heat shock response (Charng *et al.* , 2007; Jacob *et al.* , 2017; Ohama*et al.* , 2017). Considering that *HSFA2* is induced by ABA (Huang *et al.* 2016), the increased ABA content in developing Mt*hsfa9* seeds could have induced *HSFA2* and its targets. In Arabidopsis, ROF1 is thought to prolong the thermotolerance by interaction with Hsp90.1 and sustaining the level of HsfA2-regulated small HSPs (Meiri and Breiman, 2009). The ortholog of BAG6, a co-chaperone that also improves basal thermotolerance in Arabidopsis through the regulation of sHSP transcriptional cascade (Echevarría-Zomeño *et al.* 2016), was co-expressed with*MtHSFA9* (r=0.88) in seeds. *BAG6* is also a target of HSFA2 (Nishizawa-Yokoi *et al.* , 2009).

Our results show that freshly harvested seeds of *Mthsfa9* mutants germinated much slower than wild type seeds (Fig. 4). Freshly harvested wild type seeds at 40 DAP took 35 d for half of the population to germinate, whereas Mthsfa9 seeds to double the time, 69d. That this was due to a dormancy phenotype was confirmed by the removal of the phenotype by either stratification or after-ripening, leading to germination within a day (Fig. 3). Few studies link HSF or sHSPs to dormancy. A proteome study on wild *Lolium rigidum* (annual ryegrass) subpopulations selected for low and high levels of primary dormancy revealed that high-dormancy seeds showed higher abundance of sHSP than the low-dormancy seeds, and high-dormancy seeds were more tolerant to high temperatures imposed at imbibition (Goggin, Powles & Steadman 2010). Our data provide direct evidence suggesting that the role of HSFA9 is to regulate the depth of seed dormancy that is acquired during late maturation (Fig. 4d). In Arabidopsis, the depth of dormancy is regulated by DOG1 (Footitt *et al.* 2020). Interestingly in this species, *HSFA9* and its targets were downregulated in the*dog1-1* mutant and it was suggested that DOG1 activates this transcriptional cascade independently of ABI3 (Dekkers *et al.*2016). Another putative cascade in which HSFA9 could be a player in regulating depth of

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dormancy is via the endosperm expressed transcription factors ICE and ZHOUPI that act during maturation to determine the depth of primary dormancy (MacGregor *et al.* 2019). During late embryogenesis and in mature seeds, ICE1 inhibits the expression of the transcription factor *ABI3* , which itself is a central player in the formation of dormant seeds (Giraudat *et al.*1992). In Arabidopsis, ABI3 regulates HSFA9 (Kotak *et al.* 2007), and Mt*HSFA9* transcripts are also downregulated in developing Mt*abi3* mutants (Verdier *et al.* 2013). Interestingly, ICE has been found to interact with a complex including HSFA1d (Bulgakov*et al.* 2019). Here, we did not find a difference in dormancy of mature Arabidopsis *hsfa9* mutants compared to wild type (Fig. S7). However, Arabidopsis Col-0 accession seeds were not very dormant under our growth conditions, and considering that the differences in dormancy between Mt*hsfa9* and wild type were highest prior to maturation drying (Fig. 4d), our study might not have been able to reveal potential differences. Alternatively, considering the apparent absence of*DOG1* in *M. truncatula* genome, MtHSFA9 might play a more preponderant role in dormancy in *M. truncatula* than in Arabidopsis.

The dormancy phenotype of the Mt*hsfa9* mutants can be explained by an increase in ABA content in mature seeds together with an increased ABA sensitivity during imbibition (Fig. 6). This phenotype was accompanied by the deregulation of a number of genes involved in ABA homeostasis, as well as the upregulation of a putative ABA receptor (Fig. 5). Whereas it is known that ABA acts upstream of HSFAs and induces its expression (Kotak *et al.* 2007; Huang *et al.*2016; Ohama *et al.* 2017), downstream target genes of HSFAs have so far not been implicated in ABA or GA signalling. The only suggestion that ABA signalling might be affected downstream of HSFAs comes from Huang et al (2016). These authors showed that overexpression of*HSFA6* induced hypersensitivity to ABA, which is opposite of our findings. In addition, the expression of *RD22* , a drought-responsive marker gene mediated by the transcription activators MYB/MYC in an ABA-dependent manner (Abe *et al.* 2003) was unaffected in *HSFA6b* mutants but upregulated in the Mt*hsfa9* mutants (Fig. 5). The ortholog of *MYC2* in *M. truncatula* (Medtr5g030430.1) was also upregulated in the Mt*hsfa9*mutants (Data S3). Amongst the differentially expressed genes both in Mt*hsfa9* mutants and in hairy roots via ectopic expression of*MtHSFA9* (Fig. 5), we identified several genes that show a typical dormancy-related expression profile during imbibition in dormant versus fully after-ripened *M. truncatula* seeds, *MtNCED4* ,*MtCYP707A* and *MtGa2OX2* (Fig. S9). *MtCYP707A*(Medtr8g72260) is an ABA 8'-hydroxylase showing highest homology with*AtCYP707A1* , a gene that regulates ABA levels and dormancy during maturation (Okamoto *et al.*2006). Another link between HSFA9 and dormancy could be via the regulation of GA homeostasis. This is supported by the observation that GAox genes were deregulated in mature dry Mt*hsfa9* seeds. Also, RVE1 was upregulated in the mutants and downregulated in the hairy roots (Data S2). In Arabidopsis, overexpression of RVE1 lead to increased dormancy (Yang *et al.*2020). This gene directly inhibits *GA3OX2* transcription, suppressing GA biosynthesis. We were not able to alter germination speed in *Mthsfa9* seeds using paclobutrazol. While this supports a role of MtHSFA9 during maturation rather than imbibition, we cannot exclude that this GA biosynthesis inhibitor does not penetrate the seed coat (Bolingue *et al.* 2010). While this work suggests that MtHSFA9 acts as a regulator of dormancy depth, future work is needed to decipher whether HSF acts directly on genes regulating hormone metabolism and sensitivity or whether it acts indirectly through the action of specific HSPs under the control of MtHSFA9.

Short legends for Supporting Information

Figure S1. Characterization of Mt*hsfa9* mutants.

Figure S2 Identification and characterization of MtHSFA9 downstream (in)direct targets.

Figure S3. Survival curves of mature wild type (WT), associated WT (assocWT) and Mt*hsfa9* seeds from different growth environments.

Figure S4. Effect of high temperature during seed development on seed number, longevity and gene expression of HSP that are deregulated in Mt*hsfa9* mutants

Figure S5. Water content and germination curves after aging at different relative humidities (RH) of mature wild type (WT), associated WT (assocWT) and Mt*hsfa9* seeds.

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Figure S6. Deregulation of HSFA9 and two sHSP targets in Arabidopsis*hsfa9* mutants.

Figure S7. Seeds of *M. truncatula* Mt*hsfa9* Tnt1 insertion mutants exhibit a dormancy phenotype.

Figure S8. Mt*HSFA9* does not bind to the promotor of MtNCED4.

Figure S9. Differential expression of ABA synthesis, degradation, and GA inactivation genes in dormant and non-dormant *Medicago truncatula*wild type and *hsfa9* seeds.

Supplemental Data S1. Network parameters from co-expression module connecting seed-specific transcription factors to late maturation genes.

Supplemental Data S2. Differentially regulated genes in*35S::MtHSFA9* hairy roots and Tnt1 insertion mutants of

MtHSFA9

Supplemental Data S3. Differentially expressed genes in Tnt1 insertion mutants of MtHSFA9 (Medtr4g126070).

Table S1. List of primers used for experiments performed by PCR and for Yeast-One Hybrid.

Table S2. Potential heat shock element binding sites in promotors of genes that are deregulated in seeds of Mt*hsfa9* mutants.

Table S3. Gene identifiers used for the maximum parsimony analysis shown in Figure 1.

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Figure legends

FIGURE 1. Identification of MtHSFA9 as a seed-expressed gene. (a) Co-expression network of seed-specific transcription factors (squares) and transcripts correlating with the acquisition of desiccation tolerance and longevity. Node size and color represent the betweenness centrality and edge betweenness (yellow is low, blue is high). Recalculated from Verdier et al. (2013). (b) Maximum parsimony analysis of HSFA proteins from legumes (*Glycine max* ,*Medicago truncatula* , *Lotus japonica), Arabidopsis thaliana*and HaHSFA9 from *Helianthus annuus* . Gene identifiers are provided in Table S3. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates (1000) are collapsed. The tree was obtained using the Subtree-Pruning-Regrafting algorithm using MEGA7 (Kumar et al., 2016). For soybean the two paralogs are shown when appropriate. Stars indicate the *M. truncatula* ortholog of HaHSFA9. (c) Heat map of the

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HSFA family genes in *Medicago truncatula* showing mean-centered transcript levels with high (red) to low (blue) levels. Data are from the *M. truncatula* gene expression atlas (*https://mtgea.noble.org*).

FIGURE 2 . Identification of putative targets of MtHSFA9 and downstream biological processes. (a) Venn diagram between up- or downregulated genes in hairy roots overexpressing*35S::MtHSFA9::GFP::GUS* compared to hairy roots transformed with*35S::GFP::GUS* and in mature Mt*hsfa9* seeds compared to wild type seeds. (b-d) Relative transcript levels of *MtHSP17.5* (b),*MtHSP18.2* (c) and *MtHSP70* (d) in wild type seeds (WT), associated wild type seeds (assocWT) and two Mt*hsfa9* mutant alleles. Data represent the average +- S.E.M. of three technical replicates from 30 seeds. (e-h) Soluble sugar content in mature seeds of the indicated genotypes, (e) glucose, (f) sucrose, (g) stachyose, (h) verbascose. Different letters indicate significant differences between genotypes using ANOVA and post-hoc Student-Newman-Keuls comparisons (P<0.05). (i) Overrepresentation analysis of KEGG pathways of differentially expressed genes of mature Mt*hsfa9* /WT seeds.

FIGURE 3. *HSFA9* is not implicated in seed longevity under dry storage but improves survival during wet storage. (a-b) Loss of germination of mature seeds from wild type (WT) associated WT and Mt*hsfa9* plants during storage at 75% RH and 35degC (a) and at 100% RH and 40degC (b). Data are the mean (+- SE) of 3 replicates of 30 seeds. (c-d) Loss of germination of mature seeds from WT (Col-0) and*hsfa9 Arabidopsis thaliana* plants during storage at 75% RH and 35degC (c) and 100% RH and 40degC (d). At a given time point of ageing, data are significantly different when they differ by 15% or more (χ^2 test, $P < 0.05$). WT, wild type, assocWT, associated WT.

FIGURE 4 . MtHSFA9 influences the depth of dormancy during late maturation. (a) Germination curves of freshly harvested seeds of wildtype, associated WT and Mt*hsfa9* mutants. Seeds were dried for 3d at 43% RH after pod abscission before imbibition at 20°C in the dark. Data are the mean of three replicates of 30 seeds. (b) Germination curves of seeds of mature seeds from wildtype and Mt*hsfa9* mutants after stratification for 3d at 4°C. (c) Effect of after-ripening at room temperature on mean germination time of mature seeds from wildtype and Mt*hsfa9* plants grown at 20°C/18°C. Data are the mean of three replicates of 30 seeds. Stars indicate significant difference between WT and mutants using ANOVA and post-hoc Student-Newman-Keuls comparisons (P<0.05). (d) Increase in mean germination time as proxy of depth of dormancy of wildtype and *hsfa9* seeds freshly harvested at the indicated time during development. 44 DAP corresponds to the point of pod abscission. Data are the mean of three replicates of 30-50 seeds. WT, wildtype, assocWT, associated WT.

FIGURE 5 . ABA and GA biosynthesis, catabolism and signaling genes are deregulated by MtHSFA9. (a) Heatmap showing significant differential transcript levels (log2) of genes related to ABA and GA biosynthesis, reception and signaling in mature seeds of WT and two Mt*hsfa9* alleles. Asterisk indicates a significant difference in transcript level upon overexpression of Mt*HSFA9* in hairy roots. (b-d) Relative transcript levels of Mt*NCED4 (Medtr5g025270*) (b), Mt*CYP707A* (Medtr8g07226) (c) and Mt*Ga2OX2* (Medtr4g096840) (d) in mature wild type seeds (WT), associated wild type seeds (assocWT) and two Mt*hsfa9* mutant alleles. Different letters indicate significant differences between genotypes using ANOVA and post-hoc Student-Newman-Keuls comparisons (P<0.05).

FIGURE 6 . ABA content and sensitivity are affected in*Mthsfa9* mutants. (a) ABA content in mature seeds of freshly harvested associated WT and Mt*hsfa9* mutants. (b) Fold reduction in the germination speed (T50) by imbibition of mature seeds of WT, assocWT and Mt*hsfa9* seeds by 10 µM fluridone. (c) ABA sensitivity of seeds of WT, assocWT and Mt*hsfa9* mutants as measured by reduction of germination after incubation in different concentrations of ABA in the dark. (d) Time to 50% of germination or start of growth for intact scarified seeds or naked embryos of WT, assocWT and Mt*hsfa9* mutants 1 month after harvest. Seed envelopes were removed 6h after imbibition. Different letters or stars indicate significant differences between genotypes using ANOVA and post-hoc Student-Newman-Keuls comparisons (P<0.05).

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