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LARGE-SCALE BIOLOGY ARTICLE

Inference of Longevity-Related Genes from a Robust Coexpression Network of Seed Maturation Identifies Regulators Linking Seed Storability to Biotic Defense-Related Pathways

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Seed longevity, the maintenance of viability during storage, is a crucial factor for preservation of genetic resources and ensuring proper seedling establishment and high crop yield. We used a systems biology approach to identify key genes regulating the acquisition of longevity during seed maturation of *Medicago truncatula*. Using 104 transcriptomes from seed developmental time courses obtained in five growth environments, we generated a robust, stable coexpression network (MatNet), thereby capturing the conserved backbone of maturation. Using a trait-based gene significance measure, a coexpression module related to the acquisition of longevity was inferred from MatNet. Comparative analysis of the maturation processes in *M. truncatula* and *Arabidopsis thaliana* seeds and mining Arabidopsis interaction databases revealed conserved connectivity for 87% of longevity module nodes between both species. Arabidopsis mutant screening for longevity and maturation phenotypes demonstrated high predictive power of the longevity cross-species network. Overrepresentation analysis of the network nodes indicated biological functions related to defense, light, and auxin. Characterization of defense-related *wrky3* and *nf-x1-like1* (*nfxl1*) transcription factor mutants demonstrated that these genes regulate some of the network nodes and exhibit impaired acquisition of longevity during maturation. These data suggest that seed longevity evolved by co-opting existing genetic pathways regulating the activation of defense against pathogens.

INTRODUCTION

Seed maturation occurs as a set of intricate, overlapping developmental processes that occur from the end of embryogenesis until seeds become physiologically independent of the parent plant. It comprises a phase of seed storage reserve deposition and the less characterized phase of maturation drying. Furthermore, during maturation, seeds acquire a range of physiological traits that are crucial for successful seedling establishment in the field, such as vigorous and homogenous germination. These traits rely on the remarkable capacity of seeds to undergo complete desiccation without loss of viability (desiccation tolerance [DT]) and to remain alive for extended periods of time when stored in the dry state (longevity) (Buitink and Leprince, 2010). While DT is acquired during seed filling, longevity progressively increases during the final phase of seed maturation (Probert et al., 2009; Chatelain et al., 2012). Seed longevity is the ultimate survival mechanism for the plant in order to bridge unfavorable conditions. The search for the mechanisms regulating seed longevity is a longstanding goal in agriculture because of its impact on crop yields, especially in stressful conditions, and its importance as a means to preserve plant genetic resources.

So far, it has been established that the synthesis of protective molecules such as nonreducing sugars, late embryogenesis abundant proteins, and heat shock proteins is associated with longevity (Prieto-Dapena et al., 2006; Rosnoblet et al., 2007; Hundertmark et al., 2011). Furthermore, antioxidants such as glutathione (Kranner et al., 2006), tocopherols (Sattler et al., 2004), and flavonoids present in the testa (Debeaujon et al., 2000) also play a role in longevity by alleviating oxidation occurring during storage. Seed longevity is a quantitative trait for which genetic variation is observed among naturally occurring accessions and quantitative trait loci have been obtained in several crops (Bentsink et al., 2000; Nguyen et al., 2012; Nagel et al., 2015).

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These quantitative trait loci explain slight genetic variations, indicating that numerous genes influence longevity.

Several transcriptional activators have been identified that act during seed development, including the LAFL network (LEAFY COTYLEDON1 [LEC1], ABA INSENSITIVE3 [ABI3], FUSCA3 [FUS3], LEC2) and more specific regulators (e.g., VP1/ABI3-LIKE1 and HEAT SHOCK FACTOR A9 [HSFA9]) that interact with signaling hormonal responses brought by abscisic acid (ABA), gibberellins, and auxins. The LAFL transcription factor network regulates diverse seed-specific processes, including deposition of storage reserves (starch, storage proteins, and lipids), acquisition of desiccation tolerance, developmental arrest of the embryo, and dormancy (Santos-Mendoza et al., 2008). The contribution of these transcriptional activators in regulating downstream targets associated with longevity is poorly understood. To better understand how longevity is regulated during seed development, a systems biology approach provides a promising means to boost the identification of key longevity genes and their regulators.

Systems biology approaches are being increasingly used to monitor the global dynamics of entire biological circuits rather than analyzing the role of a few genes (Long et al., 2008). Genes encoding proteins that participate in the same pathway or that are part of the same protein complex are often coregulated. Therefore, clusters of genes with related functions often exhibit expression patterns that are correlated under a large number of diverse conditions. Based on the so-called "guilt by association" principle, coexpression networks have been proven to be very effective to identify relevant gene interactions (Ficklin et al., 2010; Amrine et al., 2015; Li et al., 2015) and to identify sets of candidate genes underlying specific phenotypes (Lee et al., 2009; Mutwil et al., 2010). Horvath and colleagues defined a "gene significance measure" as a means to provide a simple geometric interpretation of coexpression network analysis (Horvath and Dong, 2008). A trait-based gene significance measure assesses the relationship between a quantitative trait (such as weight) and the gene expression data. They demonstrated that the gene significance measure has great practical importance, since it allows one to incorporate external gene information into the network analysis.

Networks constructed from mixed sample sets represent a "global" or meta-analysis view of gene coexpression and have been obtained for many species (Faccioli et al., 2005; Lee et al., 2009; Ficklin et al., 2010; Schaefer et al., 2014). However, targeted networks, focusing on a specific condition, organ, or developmental phase, allow the dissection of fine regulatory switches that will otherwise be obscured through global comparisons (Usadel et al., 2009). One example is the SeedNet, a genome-wide network model describing transcriptional interactions, representing a binary phase transition study, in nongerminating (or dormant) and germinating *Arabidopsis thaliana* seeds (Bassel et al., 2011). Integration of the expression data at the resolution of developmental stages and in different environmental contexts is expected to generate a high confidence dynamic gene regulatory model (Long et al., 2008). Developing seeds represent an excellent model to test this hypothesis. In this study, we took advantage of the long maturation phase of *Medicago truncatula* seeds to create a robust regulatory coexpression network of seed maturation (MatNet). This was achieved by capturing the dynamics of 104 transcriptomes of developing *M. truncatula* seeds from plants grown under different environmental conditions. The integration of physiological data describing longevity acquisition under the different environmental conditions into MatNet, using the trait-based significance measure, led to the identification of a highly interconnected gene module related to longevity. Translation to Arabidopsis revealed a highly conserved regulatory cross-species network that was experimentally validated. Besides the identification of a number of expected functions, numerous genes in the longevity module were related to defense, suggesting that seed longevity evolved by co-opting existing genetic pathways regulating the activation of defense against pathogens.

RESULTS

Physiological and Transcriptional Profiling of Seed Traits during Development under Contrasting Environmental Conditions

To capture the effect of different environments on seed development, plants were transferred from flowering onwards to the following growth conditions: standard conditions (20/18°C), low temperature (14/11°C), high temperature (26/24°C), osmotic stress (20/18°C; soil water potential of −0.1 MPa) and greenhouse conditions (variable temperature and light). Seed development was characterized from the end of embryogenesis until final maturation drying (Figure 1). Environmental conditions strongly influenced the total developmental time, ranging from 25 to 72 days after pollination (DAP) under high and low temperature conditions, respectively (Figures 1A and 1E). Environmental conditions also affected the length of each developmental phase (embryogenesis, seed filling, and maturation drying, indicated by different colors in Figure 1F). The duration of the maturation drying phase was the most influenced (Figure 1F). Whereas the acquisition of DT appeared to be coupled with seed filling, being acquired when seed weight reached between 1 and 2 mg per seed (Figures 1A to 1E), the acquisition of seed longevity, measured as P50 (i.e., the time required for 50% of the seed lot to die during controlled storage at 35°C and 75% RH), appeared to be uncoupled from the other seeds traits. Both the duration of longevity acquisition and the final P50 were strongly influenced by environmental conditions.

In parallel to the phenotypic characterization, 104 Medicago NimbleGen arrays were analyzed for gene expression in 49 conditions resulting from different stages of development and different environmental conditions. To allow data mining of this complete data set and to aid the visualization of the gene expression data by the scientific community, a "seed" section was created in the Medicago eFP Browser that complements the already existing eFP browser framework at the Bio-Analytic Resource (http://bar.utoronto.ca/efp_medicago/cgi-bin/efpWeb.cgi?dataSource=medicago_seed). A global overview of the changes in the transcriptomes during development is presented using a principal component analysis (Figures 1G and 1H). The kinetics

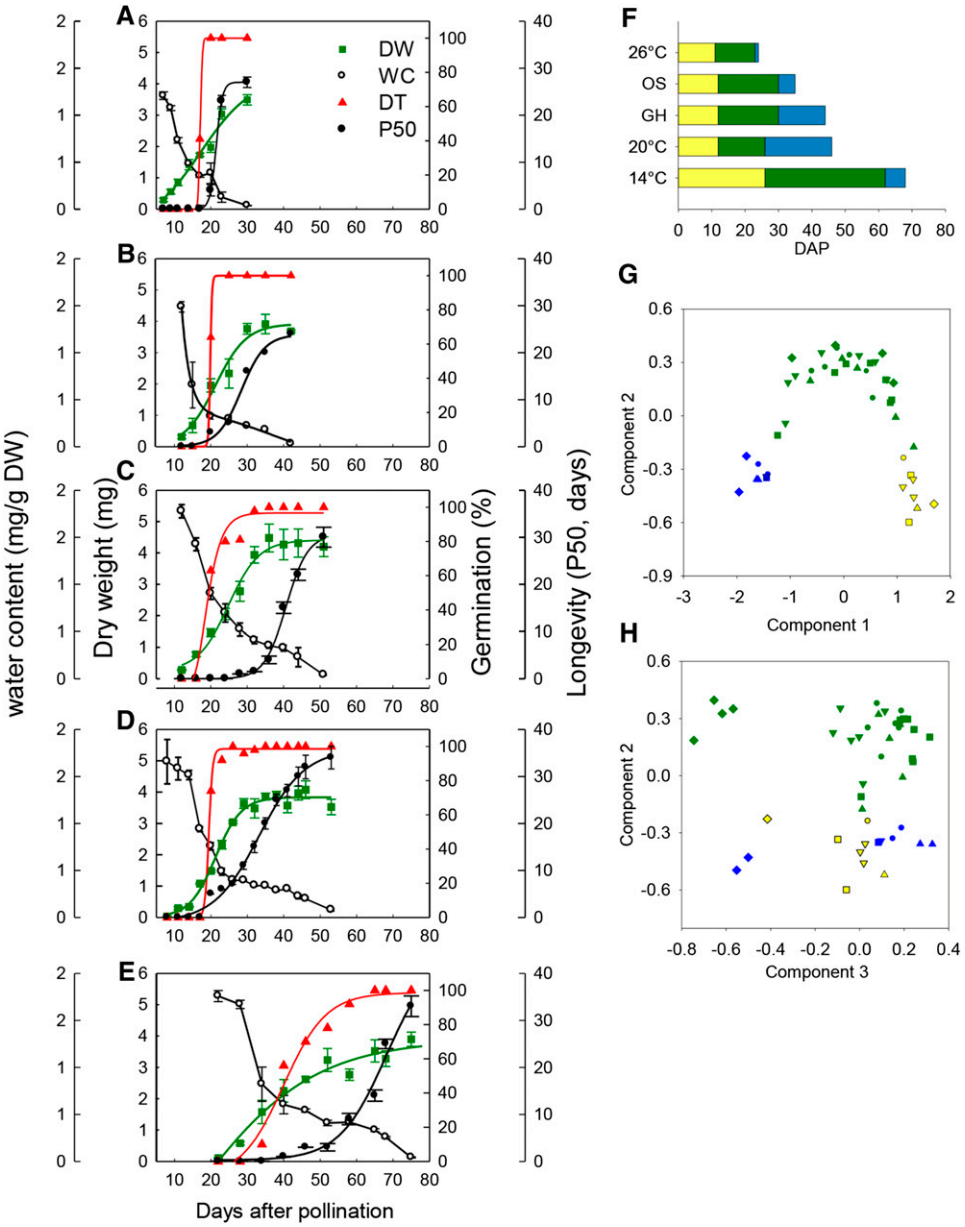

Figure 1. Seed Maturation of *M. truncatula* in Different Environmental Conditions.

(A) to **(E)** Physiological analysis describing the acquisition of quality traits (water content [white circles], dry weight [green squares], desiccation tolerance [red triangles], and longevity [P50, black circles]) in developing seeds under the following conditions: **(A)** high temperature (26/24°C), **(B)** osmotic stress (OS), **(C)** greenhouse (GH), **(D)** standard (20/18°C), and **(E)** low temperature (14/11°C) conditions. Data are means from three independent biological repetitions of 50 seeds; error bars show SD.

(F) Representation of the developmental phases in different environmental conditions: embryogenesis (yellow), seed filling (green), and maturation drying (blue).

(G) and **(H)** Principal component analysis of the transcriptome of each developmental phase (embryogenesis [yellow], seed filling [green], and maturation drying [blue]) in different environmental conditions (osmotic stress [diamonds], standard [inverted triangle] low temperature [square], greenhouse [triangle], and high temperature condition [circles]).

of seed maturation are visible in the first and second component, representing 41 and 13% of the transcriptome variation, respectively. This variation was attributed to developmental mechanisms regulating phase transitions between seed filling and maturation drying independently of the environmental conditions (Figure 1G). The third component (representing 9% of the variation) explained most of the variation observed between the different growth conditions, with the transcriptome from seeds grown under osmotic stress forming a discrete group (Figure 1H).

Generation and Characterization of MatNet: A Robust Coexpression Network of Seed Maturation

To generate a coexpression network of seed maturation, a weighted gene coexpression network analysis was performed on differentially expressed probes (Zhang and Horvath, 2005; Glaab et al., 2009). A range of edge adjacency thresholds, controlling the trade-off between coverage of coexpression associations and strength of the associations (0 = maximum coverage; 1 = maximum association strength) were tested. An adjacency threshold value of 0.88 was found to provide both sufficient coverage and association strength for further analyses and was retained for the network generation (Supplemental Figure 1A). The resulting network (MatNet) contained 2912 nodes and 736290 weighted edges (Figure 2A; Supplemental Data Set 1). The degree distribution and power law plot indicated a resulting R^2 of 0.213, which is rather low for indicating a power law typical for biological networks, but larger than for random networks (Supplemental Figure 1B) (Barabasi and Albert, 1999). However, the goal of MatNet is to provide a comprehensive collection of possible direct and indirect associations, thus focusing more on high coverage of potential interactions than on approximating the degree distribution of a typical biological network. In other words, the goal is to show all high correlations for both direct and indirect relations, not to estimate or represent direct physical interactions. The Cytoscape organic layout algorithm was used to visualize MatNet and revealed a spiral shaped network with a central cluster of interacting probes corresponding to genes with highest transcript level at the end of embryogenesis and during the seed filling phase (yellow-light green) and a tail composed of probes corresponding to genes with highest transcript level during the transition between seed filling and maturation drying phase (dark green) or increasing at final maturation (blue) (Figure 2A). The tail was connected to the central part of the network by a small number of negative connections (Figure 2A, red lines). To further characterize the network topology, transcriptional regulators were isolated from MatNet and clustered using K-means analysis on the gene expression data, revealing five main clusters (Figure 2B; Supplemental Data Set 2). These transcriptional regulators were projected onto MatNet (Figure 2C, red symbols). Using data from the Medicago Gene Expression Atlas MtGEA (He et al., 2009) and our own transcriptomic data sets (Verdier et al., 2013), we also identified probes preferentially expressed in seeds in comparison to other tissues. Out of the 2912 nodes present in MatNet, 198 had transcription levels >100-fold higher in seed versus non-seed tissues (blue symbols). Topological analysis showed that seed-specific genes were found in distinct modules (Figure 2C). The network is available for data mining at www.matnet.ml using an ad hoc interface, and the .cys file can be downloaded from https://www6.angers-nantes.inra.fr/irhs_eng/Research/Conserto.

A total of 250 transcription factors (TFs) and other transcriptional regulators (Supplemental Data Set 2) were distributed throughout the network, representing genes likely to exhibit sequential roles during seed development (Figure 2C). Relying on the high proportion of Arabidopsis homologous genes in MatNet, we further investigated the identity of the transcriptional regulators and their coregulated genes present in the different clusters of MatNet (Supplemental Data Set 2). Cluster I contained genes for which Arabidopsis homologous genes are implicated in proanthocyanidin and anthocyanin biosynthesis (*TRANSPARENT TESTA2* [*TT2*], *TT1*, *TT8*, and *ANTHOCYANINLESS2* [*ANL2*]) as well as mucilage production (*MYB86*), both processes being associated with testa differentiation during embryogenesis (Supplemental Data Set 2). Cluster II contained the master regulator *FUS3* and several TFs implicated in the regulation of seed storage compound biosynthesis (*WRINKLED1*, *LEC1-LIKE*, and *GLABRA2*) and seed size (*APETALA2*), suggesting that this cluster represents seed filling. Cluster II also contained homologs of genes encoding two Arabidopsis ABA-responsive element binding proteins (*ABSCISIC ACID RESPONSIVE ELEMENT BINDING FACTOR2* and *3*) that regulate ABRE-dependent gene expression for ABA signaling. In cluster IV, TFs were mainly related to abiotic stress such as two *DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN* genes (*DREB2F* and *DREB2D*) and a highly seed-specific *HSF* gene (*HSF30*). Finally, in cluster V, we found an Arabidopsis homolog of *DREB2C*, as well as TFs involved in flowering (putative NAC domain-containing protein 94 and *FLOWERING bHLH4*).

Identification of Gene Modules Related to Seed Longevity and Desiccation Tolerance

Coexpression networks have been proven to be effective to identify sets of candidate genes underlying specific phenotypes (Lee et al., 2009; Mutwil et al., 2010). Here, we investigated if MatNet could be used as a tool to identify gene modules linked to longevity. According to the trait-based gene significance measure (Horvath and Dong, 2008), P50 values obtained for the five developmental time series (Figure 3A) were used to identify transcripts with highly correlated expression profiles (Figure 3B). Depending on the environmental growth conditions, 2560 (in the standard condition) to 20,896 probes (high temperature condition) correlated with P50 (Pearson correlation coefficient [PCC] >0.85). A total of 498 probes were common to all environmental conditions (Figure 3B; Supplemental Data Set 3). When these probes were projected on MatNet, 167 of them formed a highly coexpressed module, representing 35% of cluster V (Figure 3C). We refer to this coexpression module as the "longevity" module (Figure 3D; Supplemental Data Set 3).

Using a similar approach of trait-based gene significance measure, we identified a "desiccation tolerance" module. For the five developmental time series (Figure 3E), transcripts were selected whose levels increased 3-fold between desiccation sensitive and tolerant stages. A total of 200 probes were common to all environmental conditions (Figure 3F; Supplemental Data Set 4). Projection on MatNet showed that 143 probes formed the desiccation tolerance module in cluster V, localized before the longevity module (Figure 3G; Supplemental Data Set 4). The DT module contained six TFs, including three APETALA2/EREBP genes (*DREB2F*), a heat shock factor *HSF30*, *TGA9*, and an unknown putative TF (Figure 3H, large symbols).

Implication of Medicago ABI3 in the Regulation of the Desiccation Tolerance and Longevity Module Nodes

In Arabidopsis, both DT and longevity are under the control of the master regulator ABI3 (Ooms et al., 1993; Sugliani et al., 2009),

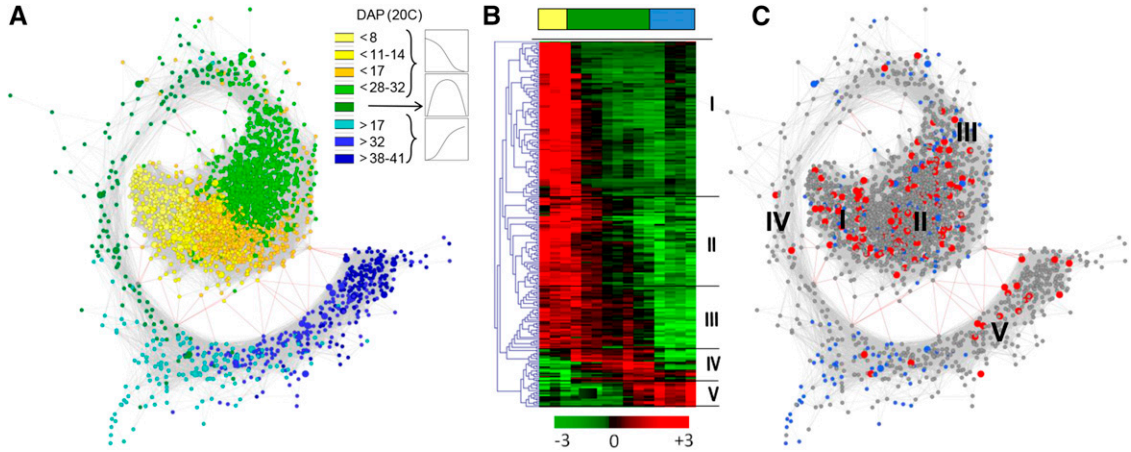

Figure 2. MatNet: A Stable Regulatory Coexpression Network of *M. truncatula* Seed Development.

(A) Weighted gene coexpression gene network of seed development of *M. truncatula*, visualized using an organic layout in Cytoscape. Temporal analysis of nodes in the network was obtained by coloring each gene by its specific expression profile along seed development. Numbers indicate the average time of seed development (in DAP) where the expression is maximum, derived from transcriptome data of seeds grown under standard conditions. Yellow-light green, high transcript levels during early seed development; dark green, transitory transcript levels; blue, transcript levels increasing during late seed development. Edges in red are negative connections.

(B) Expression profiles of TFs present in MatNet (cluster I to V).

(C) Projection of transcriptional regulators (red) and seed-specific genes (100× higher transcript levels in seed versus non-seed tissues [blue]). Expression profiles according to clusters I to V **(B)** are indicated.

suggesting that mechanisms conferring these two traits are controlled by the same regulatory pathways. However, our data demonstrate that the timing of the acquisition of longevity is much more influenced by the environmental conditions than DT (Figures 3A and 3E). This uncoupling suggests that the acquisition of these traits is controlled in part by independent regulatory pathways. To test this hypothesis and further elucidate the extent to which genes in the DT and longevity modules are under regulation of *ABI3*, we analyzed the transcriptomes of the desiccation-sensitive seeds of *M. truncatula abi3* mutants at 32 and 40 DAP (Delahaie et al., 2013; Verdier et al., 2013) (Supplemental Data Set 5). These time points coincide with an increase in the transcript levels of the longevity-correlated genes in wild-type seeds. In the DT module, 85% of the nodes were significantly upregulated (Figure 4A, red nodes) or downregulated (Figure 4A, green nodes) in developing seeds of *abi3* mutants, confirming the important role of ABI3 in the downstream regulation of these nodes. In the longevity module, 71 out of 167 probes were significantly downregulated in the *abi3* mutants either at 32 or at 40 DAP (Figure 4A, green nodes). Also, 14 probes showed transcript levels that significantly increased in the *Mtabi3* mutant at either 32 or 40 DAP (Figure 4A, red nodes). Whether the deregulation of the longevity module genes was due to a direct regulation by *ABI3* or further downstream of the ABI3 pathway was investigated through the analysis of the transcriptome of hairy roots ectopically overexpressing *M. truncatula ABI3* (GSE44291; Verdier et al., 2013). In the DT module, transcript levels of 43/143 (30%) nodes were significantly affected in the hairy roots, suggesting that their transcript levels are directly regulated by ABI3 (Figure 4A, blue nodes) (Supplemental Data Set 5). In contrast, in the longevity module, only five nodes had transcript levels that were significantly upregulated in the hairy roots. Overall, only 51% (85/167) of the probes of the longevity module were found to be affected in their expression in the *M. truncatula abi3* seeds. This suggests that at least half of the longevity module nodes appear to be regulated via alternative pathways that are not part of the ABI3 pathway (Figure 4B).

Generation of a Cross-Species Longevity Network Module of *M. truncatula* and Arabidopsis

Whereas the long maturation phase of *M. truncatula* enabled us to finely decipher those genes related to longevity, functional validation of candidate genes in longevity is tedious. Since the conserved part of the longevity regulation will enable translational biology to other species, we investigated if it was possible to identify and validate an Arabidopsis-*M. truncatula* cross-species longevity module, taking advantage of the vast amount of coexpression data of Arabidopsis. First, we characterized when DT and longevity were acquired in Arabidopsis (Figure 5A; Supplemental Figure 2). DT was acquired between 10 and 12 DAP (Supplemental Figure 2A), while longevity (P50) was gradually acquired between 12 and 17 DAP (Supplemental Figure 2B). Further maturation until 20 DAP resulted in a slight decrease in the P50. Acquisition of both traits was thus very similar for both *M. truncatula* and Arabidopsis (Figures 5A and 5B). Also depicted in Figure 5 are the principal events occurring in both species during seed maturation: accumulation of storage reserves and nonreducing sugars (data from Arabidopsis taken from Baud et al., 2002). Besides the different storage compounds, one of the major differences between Arabidopsis and *M. truncatula* seeds is that in Arabidopsis, seed filling continues until final maturation and

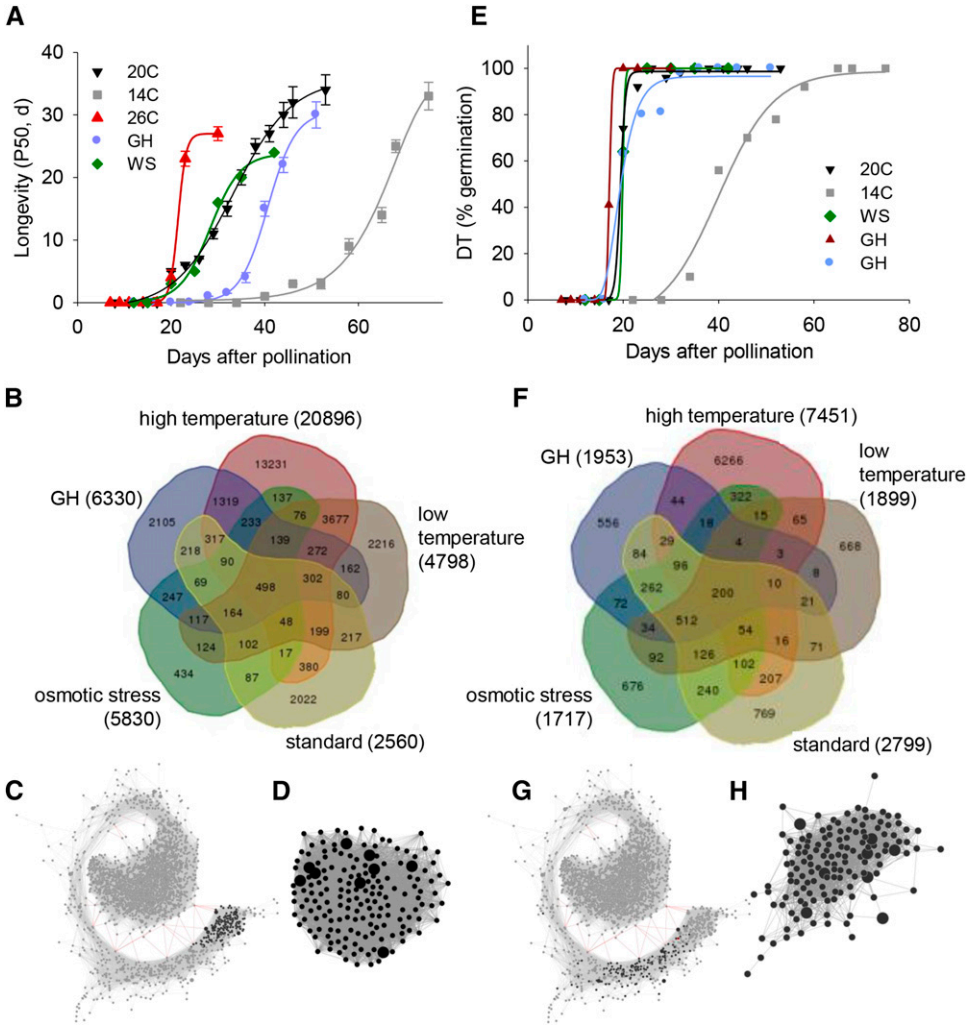

Figure 3. Identification and Characterization of the Modules Related to Desiccation Tolerance and Longevity in MatNet.

(A) The evolution of longevity acquisition, expressed as P50 (days to obtain 50% germination during storage at 35°C, 75% RH), of developing seeds under the indicated environmental conditions. Data are the average of three replicates of 50 seeds (±SE).
(B) Venn diagram of transcripts with expression profiles correlating with the acquisition of longevity (PCC > 0.85) for each environmental growth condition.
(C) Projection of the 498 probes common to all conditions in **(B)** on MatNet.
(D) Longevity module represented by the organic layout of Cytoscape. Larger nodes correspond to transcription factors.
(E) The evolution of DT acquisition, expressed as germination percentage after drying, of developing seeds under the indicated environmental conditions. Data are the average of three replicates of 50 seeds (±SE).
(F) Venn diagram of transcripts that increase I($\log_2$) > 3 upon the acquisition of DT for each environmental growth condition.
(G) Projection of the 200 probes common to all conditions in **(F)** on MatNet.
(H) DT module represented by the organic layout of Cytoscape. Larger nodes correspond to transcription factors.

overlaps with the acquisition of longevity, whereas in *M.* truncatula, both events are separated in time.

In order to identify comparable stages of seed development between Arabidopsis and *M. truncatula*, transcriptome data of different seed developmental stages were compared between both species by determination of the Spearman rank correlation coefficient (SCC) according to Patel et al. (2012). The SCC was calculated between nine seed developmental stages of Arabidopsis AtGenExpress Development Atlas (Schmid et al., 2005) and nine developmental stages of *M. truncatula* grown under standard conditions (Figure 5C). The Arabidopsis transcriptomes of the later developmental stages (stage 9 until dry seeds) overlap with a large window of *M. truncatula* development, between 23 and 44 DAP (Figure 5C). Comparison of transcript levels of marker genes of seed development also demonstrated that whereas expression of *FUS3* is transitory in *M. truncatula*, levels remain high until final maturation in Arabidopsis (Figure 5D). These observations might be explained by the continuation of seed filling processes in Arabidopsis until final maturation, whereas in *M. truncatula*, seed filling has terminated at 28 DAP (Figures 5A and 5B).

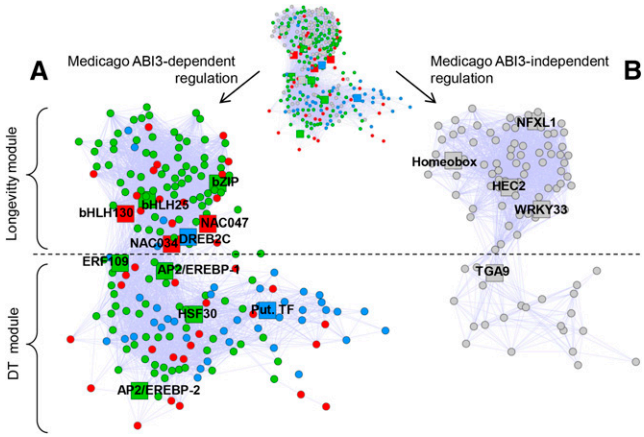

Figure 4. ABI3-Dependent and -Independent Regulation of Nodes in the Longevity and Desiccation Tolerance Module.

(A) Nodes of the longevity and DT module (Figure 3) that are significantly downregulated (green) or upregulated (red) in seeds of *M. truncatula abi3* mutants compared with wild-type seeds at 32 and 40 DAP ($P < 0.001$) or upregulated by ectopic expression in hairy roots ($\log_2 I > 1$, $P < 0.05$) (blue).
(B) Nodes of the longevity and DT module (Figure 3) that are not deregulated in the *abi3* mutants during seed development. Nodes indicated by big squares represent transcriptional factors.

Considering the overlap of the developmental programs, it was not possible to directly obtain the genes coregulating with longevity in Arabidopsis. For this reason, we used the nodes of the longevity module of *M. truncatula* and investigated whether the corresponding Arabidopsis homologous genes were connected using the extensive Arabidopsis interaction data present in Genemania (www.genemania.org). From the 167 nodes present in the *M. truncatula* longevity module, 130 Arabidopsis homologous genes were identified and submitted to Genemania. The final network contained 113 nodes and 497 edges that were mainly derived from coexpression data (431), together with 42 edges based on prediction and 23 edges based on colocalization. Thus, this analysis demonstrated that 113 genes were also connected with each other in Arabidopsis, demonstrating a high conservation of connectivity of the longevity module genes between both species (87%). Using these 113 genes, we generated a cross-species longevity network module with coregulated genes both for Arabidopsis and *M. truncatula* based on the Genemania layout (Figure 6A; Supplemental Data Set 6). A total of six TFs were present in this network (Figure 6A, large square symbols).

Validation and Characterization of the Cross-Species Longevity Network Module

To investigate the role of the network nodes in longevity, Arabidopsis T-DNA insertion mutants were phenotyped for 10 genes representing nodes of the cross-species network (Figure 6A, blue nodes; Supplemental Table 1). Genes were selected based on their elevated expression level in seeds compared with other tissues to reduce the risk of affecting plant development, thereby affecting the physiology of the seeds. No homozygous seeds could be obtained from the T-DNA insertion line of pre-mRNA polyadenylation factor FIP1 producing only 50% viable seeds, which were all wild type. Likewise, for bHLH25 mutants, only wild-type seeds were obtained and the remaining seeds were not viable, suggesting that these genes play an important role in seed development. For the other 10 genes, mutant plants were grown under controlled conditions together with the wild type (Col-0) and seeds were harvested at full maturity. No effect of the T-DNA insertion was observed on plant size or flowering time, with all plants flowering with less than a 7-d interval from Col-0. Freshly harvested seed lots from all lines showed no difference in dormancy, and after stratification, all seed lots germinated at 99 to 100% (Figure 6B, dashed line; Supplemental Figure 3). All together, these data indicate that general cellular integrity or fitness was not affected in the mutants. Next, longevity was determined on the freshly harvested seeds by controlled aging conditions (35°C and 75% RH). For most lines, germination percentage dropped faster during storage in the mutants than in the wild-type (Col-0) seeds (Figure 6B). For five out of eight genes, two mutant alleles showed significantly reduced survival during storage (Figure 6B). Complete survival curves are shown in Supplemental Figure 3.

Functional in silico characterization of the genes in the cross-species longevity module was performed exploiting the ATCOECIS online resource, which correlates Gene Ontology annotations with *cis*-regulatory elements, thereby allowing the identification of the transcriptional regulatory pathways controlling a specific set of genes (Vandepoele et al., 2009). The most representative *cis*-regulatory elements of the cross-species longevity module are involved in the response to pathogens, light, auxins, or sucrose (Table 1). Isolation and analysis of the promoters of *M. truncatula* identified similar *cis*-regulatory elements to be overrepresented in the *M. truncatula* longevity module (Table 2). Moreover, numerous genes of the cross-species network share the same *cis*-elements related to auxin, light, and defense between *M. truncatula* and Arabidopsis, suggesting a conserved regulation of the network nodes of these two species (Supplemental Data Set 6; Figures 6C to 6F). However, for the sucrose responsive binding elements, almost no overlap was found between species (Figure 6F), possibly reflecting their differential behavior in sugar accumulation (Figures 5A and 5B).

Implication of *WRKY3* and *NFXL1* in the Acquisition of Longevity during Maturation

Considering that defense is one of the overrepresented biological functions of the longevity module nodes, two TFs present in the cross-species network were further characterized because of their known role in biotic stress, *NFXL1* and *WRKY3*, the expressolog of *M. truncatula WRKY33*, which is present in the longevity module of this species. In Arabidopsis, transcripts of both genes increased during maturation and were more abundant in seeds compared with other tissues (Figures 7A and 7B; Supplemental Figure 4). Both homologous genes of *M. truncatula* exhibit similar expression profiles.

Further characterization of T-DNA insertion lines in both genes showed that dry weight and soluble sugar contents of mature seeds were not significantly different between the mutant seeds and wild-type seeds (Supplemental Figure 5 and Supplemental

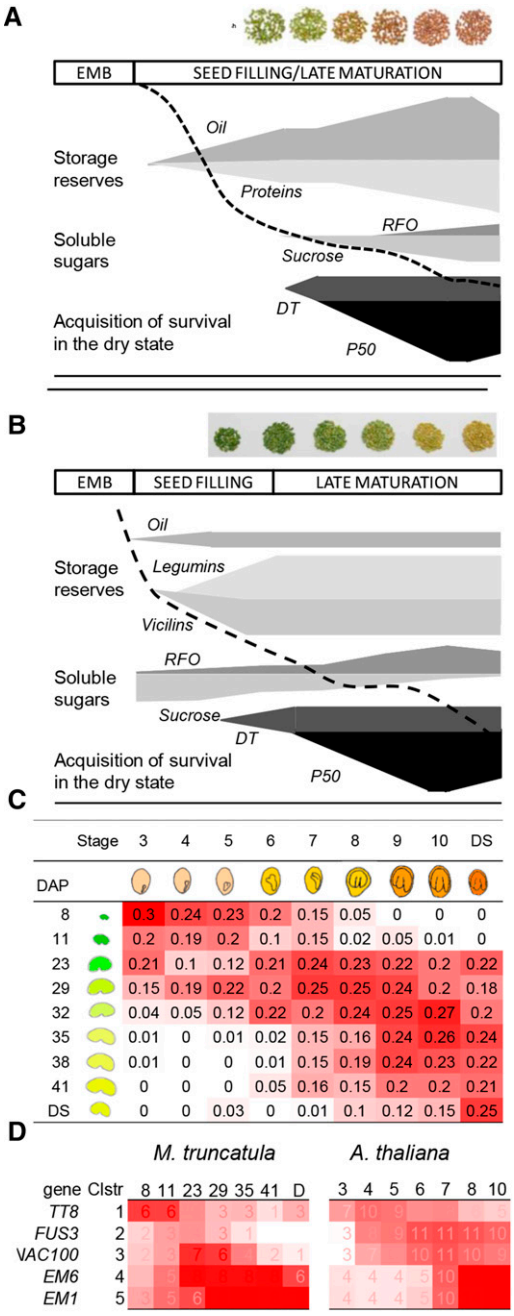

Figure 5. Comparative Analysis of Seed Maturation Events in *M. truncatula* and Arabidopsis.

(A) and **(B)** Characterization of the acquisition of different seed traits in Arabidopsis **(A)** and *M. truncatula* **(B)**. Desiccation tolerance and longevity were derived from Supplemental Figure 2 and Figure 1D. Accumulation of storage reserves and nonreducing sugars is derived from Baud et al. (2002) for Arabidopsis and from Gallardo et al. (2007) for *M. truncatula*.

(C) Heat map of SCC values between seed developmental stages of *M. truncatula* (columns) and Arabidopsis (rows).

(D) Transcript levels of marker genes of seed development in *M. truncatula* and Arabidopsis. *TT8* (*At4g09820*; *Medtr1g072320*), *FUS3* (*At3g26790*; *Medtr7g083700*), *ANAC100* (*At5g61430*; *Mtr.41161.1.S1_at*), *EM6* (*At2g40170*; *Medtr4g016960*), and *EM1* (*At3g51810*; *Medtr7g069250*). DD, dry mature seeds.

Table 2). Likewise, onset of chlorophyll loss occurred around 15 DAP, without any apparent difference for the *nfxl1* and *wrky3* mutants compared with wild-type seeds (Figure 7C). At 15 DAP, longevity (P50) of wild-type seeds has been acquired for ~40% compared with the mature wild-type seeds (Figures 7D and 7E). The acquisition of longevity was already affected in both *wrky3* and *nfxl1* mutants seeds that were harvested at 15 DAP (Figures 7D and 7E, dashed lines). This longevity difference between mutant and wild-type seeds increased slightly upon further maturation. Furthermore, ABA sensitivity was not significantly affected in mature seeds of both mutants (Figure 7F), albeit that there was a slight significant increase in sensitivity for seeds of the *wrky3-1* line (Figure 7G).

To explore the predictive role in of the edges in the cross-species network in mediating gene-gene interactions, we investigated whether the nodes in the cross-species network were under the regulation of WRKY3 or NFXL1 by qPCR. Analysis of the presence of W-box and/or NF-x putative *cis*-regulatory elements in the promoter genes of the longevity module revealed that 12 of them contained both *cis*-elements, including *HSPRO2* and *WRKY33* and are first neighbors of both *WRKY3* and *NFXL1* in the cross-species network (Supplemental Data Set 6). In *nfxl1* mutant seeds at 15 DAP, transcript levels of *HSPRO2* were significantly lower than in wild-type seeds (Figures 7H, II). In 15 DAP seeds of *wrky3* mutants, transcript levels of both *HSPRO2* and *WRKY33* were affected (Figure 7I, I and II). Promoter analysis of *NFXL1* and *LZF1* genes revealed the presence of the W-box element. *NFXL1* transcripts were lower in the *wrky3* mutants (Figure 7I, III) whereas no difference was found in the transcript levels of *LZF1* in either of the mutants (Figures 7H and 7I, IV).

In Arabidopsis, seeds of mutants with defects in seed coat properties such as permeability and composition such as flavonoid pigmentation exhibit reduced storage stability (Debeaujon et al., 2000). Flavonoid pigmentation is controlled by several *tt* genes, some of which were found in our cross-species longevity (Supplemental Data Set 6). This prompted us to investigate whether the testa was affected in mature *nfxl1* and *wrky3* seeds. Seed coat permeability was evaluated in mature seeds by incubation in tetrazolium salts for 12 h. In a repeatable manner, the number of stained seeds for both the *nfxl1* lines (Figures 7J and 7K) and *wrky3* lines (Figures 7J and 7L) was doubled compared with the corresponding wild-type seeds.

DISCUSSION

Translational Biology between *M. truncatula* and Arabidopsis Infers a Cross-Species Gene Module Related to Longevity

The construction of MatNet, a robust coexpression network of seed maturation, and the subsequent integration of the acquisition of longevity using trait-based gene significance measure (Horvath and Dong, 2008) led to the discovery of a gene coexpression module from which confirmed and new genes regulating seed longevity were successfully inferred. MatNet is based on the reasoning that although environmental growth conditions will affect the timing and extent of different seed traits that are

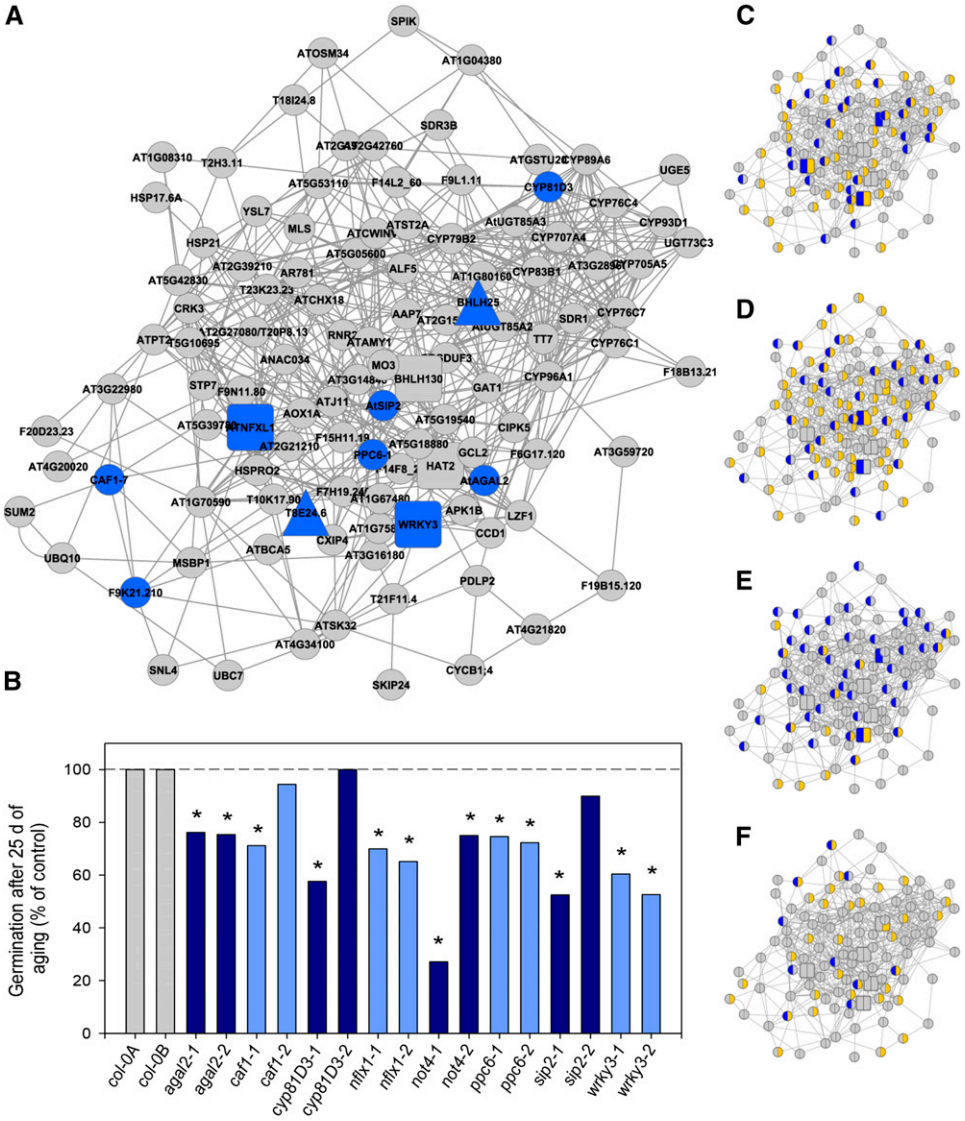

Figure 6. Identification and Characterization of Cross-Species Longevity Network Module.

(A) Regulatory gene network with nodes representing both Arabidopsis and *M. truncatula* homologs that are coregulated in relation to longevity.
(B) Genes of colored nodes in **(A)** were functionally analyzed for longevity phenotype by comparing germination after 25 d of aging between mutant and wild-type seeds. The dashed line corresponds to the germination percentage of freshly harvested, non-aged seeds. The asterisk indicates a significant difference from the wild-type Col-0 seeds (χ^2 test, $P < 0.001$).
(C) to **(F)** Genes with overrepresented *cis*-elements in *M. truncatula* (blue) and Arabidopsis (yellow) promoters; **(C)** response to auxin stimulus, **(D)** light regulated, **(E)** defense response to pathogen, and **(F)** SURE, sucrose binding element.

acquired during seed development, the genes that are part of the core genetic pathways governing the establishment of these traits will always be expressed. The integration of transcriptomes of developing seeds obtained from five growth conditions into the network led to the separation of functionally associated genes from those coexpressed due to overlapping developmental processes or sampling differences, thus removing biological noise and capturing the conserved backbone of seed maturation. As such, MatNet displays a higher level of internal network structure compared with the structure of a network based on a single environmental condition (Verdier et al., 2013). In addition, MatNet was successful in inferring regulatory genes controlling seed longevity due to the long maturation time frame of *M. truncatula* seeds, which allowed detailed mapping of physiological events from seed filling onwards (Verdier et al., 2013). In Arabidopsis, the short maturation phase results in substantial overlap between seed filling and late maturation, complicating the discrimination between processes regulating longevity and other traits (Figure 5). Indeed, in this species, a total of 8329 genes correlated with longevity (PCC > 0.85), including *FUSCA3* and *ANAC100*, for which the *M. truncatula* homologs are expressed transitorily during maturation and do not correlate with longevity.

Table 1. Analysis of Significant *cis*-Regulatory Elements of the Genes Present in the Longevity Cross-Species Arabidopsis-*M. truncatula* Module

GO Categories Enriched with Motif (P Value < 0.001)	Motif	P Value	Enrichment Fold
Oxygen binding, transmembrane movement	ANTATTTC	3.88E-05	27
Oxygen binding, defense to pathogen	NGNACATA	3.60E-04	24
Defense response to pathogen	AANGTCAA	4.19E-04	19
Light-regulated genes	NNTGGATA	6.33E-04	34
Glucosyltransferase	AGAATATN	6.88E-04	21
Carboxylic acid biosynthesis	TANACGNA	1.30E-03	19
Carbohydrate biosynthesis	AANGNCTG	2.53E-03	15
Golgi apparatus	AGATCTNN	3.45E-03	18
Response to auxin stimulus	CTATATAN	3.46E-03	22
SURE2STPAT21	AATACTAAT	4.38E-03	10
Response to auxin	ATANAGAG	5.39E-03	17
Defense response biotic	AAGTCNAC	6.00E-03	7
Response to wounding	ATATATNC	7.14E-03	21
Response to wounding	CGNNTATA	9.27E-03	14

ATCOERCIS Web-based resource was used to determine *cis* elements enriched motifs using on the −1000 bp upstream of Arabidopsis promoters.

The subsequent translation of MatNet to Arabidopsis shows a high level of connectivity (87%) of the longevity module genes both for *M. truncatula* and Arabidopsis, suggesting conserved genetic pathways regulating longevity. This suggestion is also evident from the analysis of the promoter regions of the cross-species longevity genes showing common *cis*-regulatory elements related to auxin, light, and to a lesser extent biotic defense responses in both species (Figure 6). The main difference was found for sugar responsive elements. This might be related to the different sugar metabolism that takes place during final maturation. In developing seeds of *M. truncatula*, sucrose levels decrease to 10% of the total soluble sugar content in mature seeds, at the expense of raffinose family oligosaccharide accumulation, whereas in developing seeds of Arabidopsis, sucrose levels increase until final maturation and represent 80% of the total soluble sugars (Figure 5).

Functional validation of the genes inferred from the cross-species longevity module identified with a high success rate (5/8) new and confirmed genes controlling seed longevity. The longevity module contained a number of genes implicated in previously identified mechanisms conferring longevity, such as DNA repair (*DNA LIGASE-1*) and protection by the testa pigments (*TT7* and mate efflux family protein with high sequence similarity to *TT12*). Also, the presence of several HSPs (*HSP17.6*, *HSP21*, and *DnaJ*) is in accordance with previous studies demonstrating the importance of these proteins for seed longevity (Almoguera et al., 2009). The identification and further characterization of an α-galactosidase, *AGAL2*, for which mutant seeds showed decreased longevity and increased sucrose and stachyose contents (Supplemental Figure 6) points to a role of raffinose family oligosaccharide metabolism in longevity. Nonreducing sugars are associated with survival in the dry state, although a direct role in the acquisition of longevity remains to be proven. In our study, the increased amounts of nonreducing sugar molecules in the mutant seeds cannot explain the reduced longevity via a protective function and confirmed previous genetic and biochemical studies (Bentsink et al., 2000; Buitink et al., 2000).

Several lines of evidence suggest that there is an ABI3-independent pathway regulating the acquisition of longevity. Half of the longevity nodes were not altered in transcript levels in the *M. truncatula abi3* seeds. ABI3, together with other members of the LAFL network, is implicated in the cellular phase transition from the embryonic to the vegetative stage, regulating major developmental changes related to seed storage accumulation and DT (Santos-Mendoza et al., 2008). In Arabidopsis, weak alleles of *ABI3* are fully desiccation tolerant but have reduced storability, making it one of the major regulators of longevity (Ooms et al., 1993; Sugliani et al., 2009; Verdier et al., 2013). In *wkry3* and *nfxl1* mutant seeds, dry weight accumulation and other developmental processes regulated by ABI3, such as chlorophyll degradation and the accumulation of nonreducing soluble sugar contents, were not affected in mature seeds, and *NFXL1*, *WRKY3*, or *WRKY33* have not been identified as targets of ABI3, LEC1, or LEC2 (Braybrook et al., 2006; Junker et al., 2012; Mönke et al., 2012). In *M. truncatula*, our transcriptome data on *abi3* mutant seeds and ectopic expression of *ABI3* using a hairy root system demonstrate that *NFXL* and *WRKY33* transcript levels were not deregulated by ABI3, making these TF putative regulators of an ABI3-independent pathway involved in longevity.

A Role for Auxin in the Regulation of Longevity

Surprisingly, genes that are present in the cross-species longevity module are highly enriched in binding sites for auxin response

Table 2. Analysis of Significant *cis*-Regulatory Elements of the Genes Present in the Longevity Module of MatNet

Description	*cis*-Element	Sequence	P Value	O	E	N
Auxin-responsive factors	ARFAT	TGTCTC	1.28E-112	59.62	6.33	15
Light response promoter regions	IBOX	GATAAG	5.98E-103	57.69	6.42	28
Phosphate starvation-responsive gene	P1BS	GNATATNC	2.11E-071	36.54	3.72	9
ABA induced	DRE2COREZMRAB17	ACCGAC	3.20E-049	25.00	2.53	7
Pathogen-responsive genes	GCCCORE	GCCGCC	2.52E-042	17.31	1.46	1
Gibberellin-responsive element	TATCCACHVAL21	TATCCAC	1.48E-035	19.23	2.07	1
Sucrose-responsive element	SURE1STPAT21	AATAGAAAA	5.10E-031	17.31	1.92	2

O and E, observed and expected frequency in the longevity module; N, number of promoters whose Z score was >1.5. *cis*-elements were determined using the PLACE Web-based resource on the −1000 bp upstream of *M. truncatula* promoters.

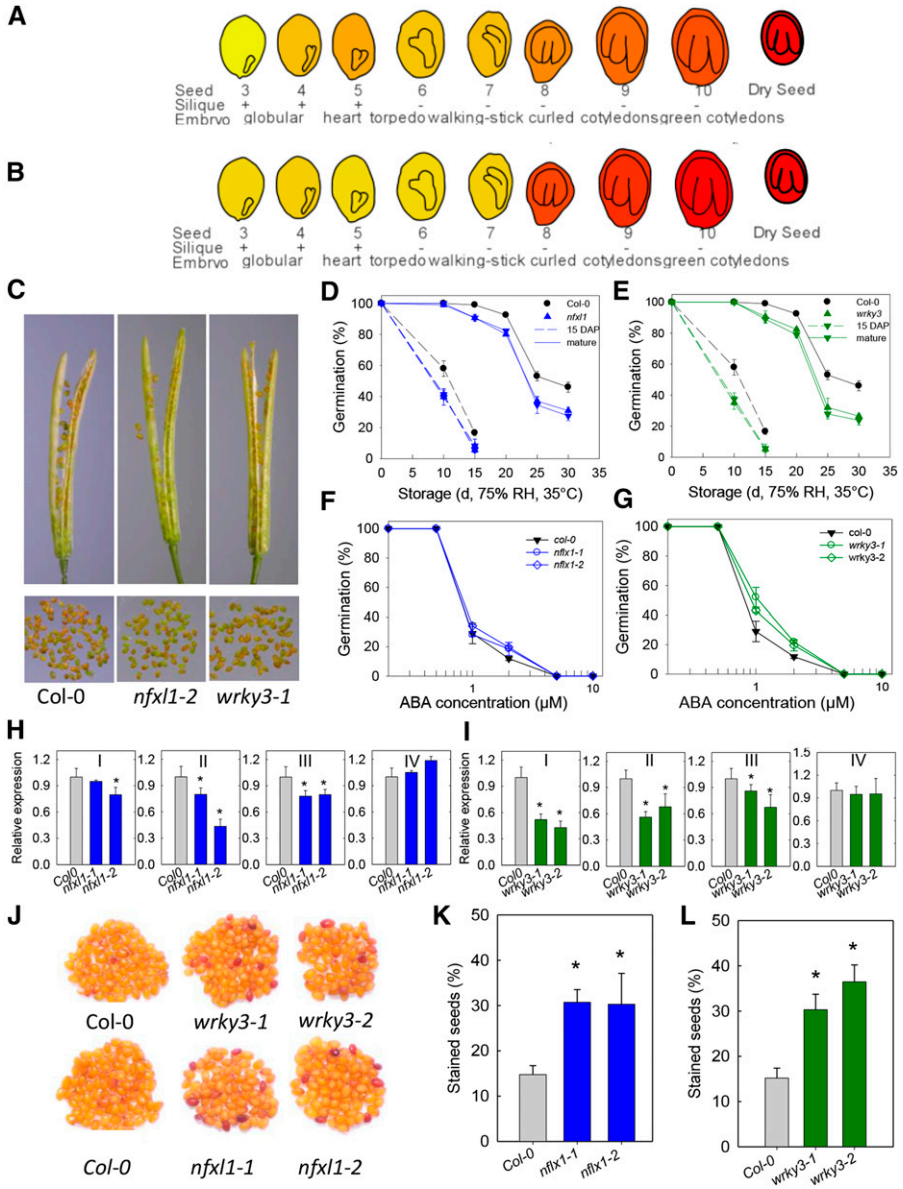

Figure 7. Characterization of *nfxl1* and *wrky3* Mutants in Seed Maturation and Longevity Acquisition.

(A) Absolute expression of *NFXL1* (At1g10170) in Arabidopsis seed development.
(B) Absolute expression of *WRKY3* (At2g03340) in Arabidopsis development. Data are taken from the eFP browser 2.0 (www.bar.utoronto.ca).
(C) Representative picture of siliques and seeds of mutants and the wild type harvested at 15 DAP.
(D) and **(E)** Loss of germination during storage (75% RH, 35°C) of seeds at 15 DAP (dashed lines) or at maturity (solid lines) for the wild type, *nfxl1* **(D)**, and *wrky3* **(E)**.
(F) and **(G)** Germination of *nfxl1* **(F)**, *wrky3* **(G)**, and wild-type seeds after 14 d of incubation in ABA. For **(D)** to **(G)**, 100 seeds of three biological replicates were tested. Germination between lines is significant when >8% (χ^2 test, $P < 0.05$)
(H) and **(I)** Transcript levels of four network nodes: *WRKY33* (I), *HSPRO2* (II), *NFXL1* (III), and *LZF1* (IV). The asterisk indicates a significant difference from the wild-type Col-0 seeds (*t* test, $P < 0.01$). Levels in *nfxl1* mutants **(H)**; transcript levels in *wrky3* mutants **(I)**.
(J) Wild-type, *nfxl1*, and *wrky3* seeds after 12 h of tetrazolium staining.
(K) and **(L)** Number of stained (red) seeds in wild-type and *nfxl1* **(K)** and *wrky3* **(L)** seeds, determined on three biological replicated of 50 seeds. The asterisk indicates a significant difference from the wild type Col-0 seeds (χ^2 test, $P < 0.001$).

factors (ARFs) in their promoters (Supplemental Data Set 3). Instead of an expected 6% of genes, 60% of the *M. truncatula* genes of the longevity module contain the ARFAT *cis*-element (Table 2). Previously, a link between auxin and longevity was established by Carranco et al. (2010), who showed an interaction between the auxin/indole-3-acetic acid protein IAA27 and the heat shock factor HSFA9 of *Helianthus annuus*. HSFA9 activates transcription of specific small heat shock protein (sHSP) genes and is involved in the control of a genetic program that regulates seed longevity (Prieto-Dapena et al., 2006; Almoguera et al., 2009). In our study, ARF binding sites were found in the promoters of the *M. truncatula* and Arabidopsis *HSP21* of the longevity module. An increase in auxin in maturing seeds might result in a derepression of the *sHSP21* genes, possibly in interaction with HSFA9, although further work is needed to corroborate this hypothesis. Further analysis of the genes with *cis*-regulatory ARF elements points to a role of the degradation of Trp upstream of the indole-3-acetic acid (IAA) and indole glucosinolate biosynthetic pathways (Supplemental Data Set 6). Four nodes in the longevity module are linked to this degradation pathway: *CYP79B2*, which converts Trp to indo-3-acetaldoxime, a precursor of both IAA and indole glucosinolates (Kim et al., 2015); *CYP83B1* (*SUPERROOT2* [*SUR2*]), an enzyme in the metabolic branch point between IAA and indole glucosinolate biosynthesis, regulating IAA homeostasis (Bak et al., 2001); *CYP76C7*, involved both in the Trp catabolic process and regulating *CYP79B2* and *SUR2*; and *CYP82C2*, shown to modulate indole glucosinolate biosynthesis as well as jasmonate-induced root growth inhibition and defense gene expression (Liu et al., 2010).

Link between Seed Longevity and Genetic Pathways Regulating the Activation of Defense against Necrotrophic Pathogens

Another surprising finding inferred from the cross-species network is the high number of genes related to defense against pathogens that are present in the longevity module. Several lines of evidence support this previously unidentified link between defense-associated genes and longevity. First, mutation of *WRKY3* and *NFXL1*, two TFs previously implicated in defense against pathogens, led to reduced longevity. WRKY3 is a homolog of the *M. truncatula* WRKY33, present in the longevity module of this species. Although At-WRKY33 has a slightly higher sequence similarity to Mt-WRKY33, we chose to investigate *WRKY3* since its expression is much more similar to that of Mt-*WRKY33* than At-*WRKY33*, as both genes are highly expressed at the end of seed development. Interestingly, we found that transcript levels of *WRKY33* decreased in the *wrky3* mutants, indicating that these genes are part of an intricate regulatory network. After infection with the necrotrophic fungal pathogen *Botrytis cinerea*, leaves of *wrky3* mutants exhibit more severe disease symptoms and support higher fungal growth than wild-type plants, whereas no effect is detected with the biotrophic pathogen *Pseudomonas syringae* (Lai et al., 2008). Likewise, the *WRKY33* transcription factor is required for resistance to necrotrophic fungal pathogens concomitant with reduced expression of the jasmonate-regulated plant defensin gene *PDF1.2* (Zheng et al., 2006). It should be noted that the *M. truncatula* homolog of *PDF1.2* is part of the *M. truncatula* longevity module (Supplemental Data Set 3), although it was not retained in the cross-species longevity module. As inferred by the network, seeds of *wrky3* mutants are affected in the acquisition of longevity and also exhibit a higher permeability of tetrazolium salt (Figure 7), suggesting defects in the testa (Debeaujon et al., 2000).

A second transcription factor that is part of the cross-species longevity network is NFXL1, a NF-X1-type zinc finger protein. We demonstrated that this transcription factor is also involved in seed longevity and seed coat permeability. As for *WRKY3*, its expression is strongest in seeds, with an increase during final seed maturation (Figure 7). In Arabidopsis, *NFXL1* has been implicated in the trichothecene phytotoxin-induced response as well as in the general defense response (Asano et al., 2008). In addition, plants overexpressing *NFXL1* display a higher survival when subjected to abiotic stresses such as salt, drought, or high light intensity (Lisso et al., 2006).

A search for W-box and NF-x putative *cis*-elements in the promoter region of the cross-species longevity module nodes revealed several putative targets for WRKY3 and NFXL1, such as *CYP79B2* and *CYP83B1*, the two genes implicated in auxin biosynthesis and homeostasis (Supplemental Data Set 6). Here, we experimentally confirmed that *NFXL1* transcript levels are deregulated in seeds of *wrky3* mutants harvested at 15 DAP, demonstrating the predictive power of the cross-species longevity module. In addition, *HSPRO2* transcripts were lower in both *nfxl1* and *wrky3* mutant seeds. HSPRO2 is a positive regulator of basal defense against pathogens, as Arabidopsis knockout mutants are more susceptible to *P. syringae* infection (Murray et al., 2007). HSPRO2 interacts with AKINβγ, a subunit of the Sucrose nonfermenting-Related Kinase complex (SnRK1) (Gissot et al., 2006). SnRK1 functions as a central integrator of transcription networks in plant stress and energy signaling, in particular in response to biotic and abiotic stress (Schwachtje et al., 2006; Baena-González et al., 2007). It has been suggested that HSPRO2 participates in the activation of SnRK1-mediated response to regulate changes in metabolism required for defense against pathogens. For example, HSPRO2 and GAL83, another subunit of the SnRK1 complex, are part of the same regulatory pathway, negatively regulating seedling growth during interaction with *Piriformospora indica* (Schuck et al. (2013). In a previous study (Rosnoblet et al., 2007), we demonstrated that silencing SNF4b, a seed-specific regulatory subunit of the SnRK1 complex in *M. truncatula*, results in reduced longevity. Interestingly, *SNF4b* regulates defense gene expression in dormant *M. truncatula* seeds during imbibition such as *ABR18* and *WRKY33*, which are also present in the *M. truncatula* longevity module (Bolingue et al., 2010).

The presence of the defense genes in the cross-species longevity module, as well as genes implicated in the auxin and indole glucosinate biosynthesis, points to a role of passive, developmentally regulated biotic defense in the acquisition of longevity during late seed maturation. This developmentally regulated biotic defense appears to employ similar pathways as those used upon infection with necrotrophic pathogens. Mechanisms involved in passive defense include physical barriers related to thickened cell walls, or substances such as cutin, suberin, or pectin and chemical barriers, including the accumulation of compounds with microbial activity such as phytoanticipins (constitutively regulated phytoalexins), defensins, saponins, or

certain defense proteins (Walters, 2010). That a preemptive strategy to protect seed embryos from pathogen attack is also serving a function in seed physiology has received little attention and is so far limited to the observation that the thick layer of β-glucans, which can defend plants against infection, also play key role in regulating seed coat-imposed dormancy (reviewed in Leubner-Metzger, 2003). In our study, the increased permeability of the *wrky3* and *nfxl1* seeds indicates a difference in the physical barriers that might explain the longevity phenotype. Other mutants with increased seed coat permeability that are also known to be affected in seed longevity are the *tt* mutants, functioning in the synthesis of the flavonoids anthocyanins and proanthocyanidins (Debeaujon et al., 2000; Clerkx et al., 2004). One of the major functions of these molecules is to provide protection against microbial pathogens, insect pests, and herbivores (Dixon, 2005). Recently, a study conducted on *M. truncatula* revealed the activation of *tt* gene expression during bacterial infection of seeds (Terrasson et al., 2015), suggesting the existence of a complex regulatory interplay involved in seed coat and stress response. The longevity module contains two *tt* genes (TT7 and a MATE efflux family protein), although transcript levels of *TT7* were not deregulated in a consistent manner in the *wrky3* and *nfxl1* seeds (data not shown). Alternatively, there is compelling evidence that auxins play a role in the resistance to necrotrophic pathogens via modulation of development such as cell wall properties (Raiola et al., 2011). The presence of W-box and NF-x regulatory *cis*-elements in the promoter regions of IAA regulating genes *CYP79B2* and *CYP83B1* and the high number of ARFs in the longevity module provide another link between pathogen-related and developmental roles of auxins and offer a new research area for seed biology. Across 195 species, seed longevity is correlated to seed structure and climate origin but not with seed mass and storage composition, with early angiosperms having short-lived seeds in dry storage (Probert et al., 2009). We hypothesize that seed longevity has evolved by co-opting existing genetic pathways regulating the activation of defense against necrotrophic pathogens to ensure prolonged survival in the dry state to be ready for next generations.

METHODS

Plant Material and Physiological Analyses

Plants of *Medicago truncatula* Gaertn (A17) were grown in standard conditions (20°C/18°C, 16-h photoperiod, according to Verdier et al. [2013]). At the start of flowering, plants were maintained in these conditions or transferred to different environmental conditions: high (26°C/24°C, 16 h) or low (14°C/11°C, 16 h) temperature, osmotic stress (20°C/18°C, 16 h), and maintaining a soil water potential at −0.1 MPa, greenhouse (16 to 30°C, variable photoperiod). Flowers were tagged and developing seeds were harvested at different time intervals until pod abscission and after final desiccation. Water contents were assessed gravimetrically for triplicate samples of five seeds by determination of the fresh weight and subsequent dry weight after 2 d in an oven at 96°C. Water contents are expressed on a dry weight basis. DT and longevity were determined according to Chatelain et al. (2012) on three biological replicates of 50 seeds that were rapidly dried at 43% RH under airflow and harvested from a pool of three plants each. Longevity was determined for each of the 49 developmental stages of each environmental growth condition as the storage time that was needed to decrease the germination of the seed population to 50% (P50), derived from survival curves of percentage of final germination over storage time (75% RH, 35°C). The *M. truncatula abi3* mutant (NF6003) was isolated and backcrossed twice with wild-type plants as described by Delahaie et al. (2013). Seeds of *abi3* mutants and the wild type (R108) were collected at 40 DAP and frozen for transcriptome analysis.

Plants of *Arabidopsis thaliana* (Col-0) were grown at 20°C/18°C with a 16-h photoperiod. Flowers were tagged and developing seeds were harvested at different time intervals until pod abscission and after final desiccation. After rapid drying under airflow at 43% RH, DT and longevity were determined as described above. Arabidopsis T-DNA insertion mutants (Supplemental Table 1) were obtained from the Nottingham Arabidopsis Stock Centre, and homozygous seeds were identified by PCR (for primer details, see Supplemental Table 3). Plants were grown together in the same conditions for the production of seeds. The onset of flowering and plant height was recorded for each genotype. Flowers were tagged every day, and seeds were harvested at 15 DAF and fully mature and rapidly dried for 3 d at 43% RH under constant airflow. Dry weight was assessed gravimetrically for triplicate samples of 25 seeds by determination of the weight after 2 d in an oven at 96°C. Physiological experiments were performed on three biological replicates of 100 seeds, harvested from a pool of three to six plants. Viability of the freshly harvested seeds was determined by germination after imbibition of seeds for 2 d at 4°C to remove dormancy, followed by incubation on Whatman filter paper No. 1 in 9-cm Petri dishes with 1 mL water at 20°C, 16 h light. Longevity was determined as described above. To determine ABA sensitivity, mature seeds were imbibed on filter paper on a range of ABA concentrations (mixed isomers; Sigma-Aldrich) at 20°C, 16-h light. ABA was dissolved in methanol prior to dilution in water. Control seeds were imbibed in the methanol concentration corresponding to the highest ABA concentration (0.05% methanol). Germination was scored after 14 d.

Permeability of the seed layers was assessed by incubation of three biological triplicates of 50 seeds consisting of a pool of six to eight plants each in 1% 2,3,5 triphenyltetrazolium chloride in 50 mM phosphate buffer (pH 7.0) for 12 h at 30°C, after which image analysis and percentage of red colored seeds were counted.

Transcriptome Acquisition, Data Analysis, and Bioinformatics

RNA extraction, amplification, labeling, hybridization on NimbleGen slides, and statistical data analysis were conducted as described by Verdier et al. (2013). For seeds of the 49 different developmental stages and growth conditions, two biological replicates of 50 seeds harvested from a bulk of five different plants and immediately frozen in liquid nitrogen after pod dissection were analyzed per comparison using dye switch. For the 40 DAP *M. truncatula abi3* seeds, three biological replicates were used. Transcriptome data have been submitted to the Gene Expression Omnibus as SuperSeries GSE53526, containing the following subseries: 21/19°C (GSE49350), 26/24°C (GSE52832), 14/11°C (GSE53002), under greenhouse conditions (GSE52833), and osmotic stress (GSE53003); data on the ectopic expression of Mt-*ABI3* in hairy roots are submitted under number GSE44291, and the *abi3* mutant versus wild type transcriptome as GSE57457.

For *cis*-element enrichment analysis of *M. truncatula* promoters, DNA motifs were counted (using PLACE database resource, http://www.dna.affrc.go.jp/PLACE/signalup.html) within the region 1.5 kb upstream of the translational start site based on *M. truncatula* IMGAG 3.5.1 version using the Legoo Gateway (https://www.legoo.org/). The frequency of a motif in the promoters of genes of the longevity module (105 genes for which the promoter sequence was retrieved) was compared against a background frequency generated using the total genes present in MatNet (1624 genes for which the promoter sequence was retrieved). P value significance scores were calculated using a χ^2 test to provide a relative ranking. An enrichment analysis was performed based on the number of elements present in the same promoter. The Z-score was calculated for each gene in the longevity module based on (X-mean)/SD,

where X is the number of repetitions of a particular *cis*-element, and mean and SD are the average and the SD, respectively, of the element repetition in MatNet promoters. Analysis of promoter elements/Gene Ontology in Arabidopsis was conducted using the ATCOECIS online resource (http://bioinformatics.psb.ugent.be/ATCOECIS/), which determines both Gene Ontology and motif enrichment. The sequence of the NF-x regulatory element was derived from Song et al. (1994).

eFP Browser Visualization

For the integration of the Nimblegen transcriptomic data with the already existing data used for the eFP browser visualization, data were RNA-normalized and then linearized, being scaled such that expression levels of guide genes known to be involved in different stages of seed development (*PHB*, *ATB2*, *AP3*, *SEP2*, *MYB3R1*, *ABI5*, *ABI3*, *AREB3*, *TT8*, and *HAP3*) were consistent with the previous eFP browser view.

Identification of Seed-Related Genes

To identify seed related or seed specific probe sets, a Z-score was calculated using Z = (X-mean)/SD, where X is the relative expression value, mean is the average of relative expression of tissues available in MtGEA (http://mtgea.noble.org/), and SD is the SD of relative expression in all tissues available in the MtGEA. Probe sets with a Z-score above 2.0 in seeds were considered as seed-specific or preferentially expressed in seeds.

Network Generation and Inference of Desiccation Tolerance and Longevity Modules

The gene coexpression network MatNet was constructed from 104 microarray data sets corresponding to 49 developmental stages of *M. truncatula* seeds grown in the five different environmental conditions. Only those probes with variance >0.1 across all samples were retained to filter out genes with largely unchanged expression levels. Weighted coexpression network analysis (Zhang and Horvath, 2005) was performed using the ArrayMining Web resource (http://www.arraymining.net) (Glaab et al., 2009). The number of nodes and edges were determined for each adjacency threshold ranging from 0.8 to 0.94 with 0.2 intervals. Gene interactions were visualized using the open source software Cytoscape (version 2.8.1) using an organic layout.

Isolation of the longevity module was performed using the trait based gene significance measure, obtained by

$$GSi = \left|cor(xi, P50)^{\beta}\right|$$

where the gene significance (GS) of gene i equals the absolute correlation between the gene expression profile xi and longevity expressed as P50 (days to obtain 50% germination during storage), with $\beta = 1$ (Horvath and Dong, 2008). Modulating the PCC to 0.9 or 0.8 changed the number of probes that correlated with P50, but after projection of these data lists on MatNet, the same 167 probes were identified. Equally, raising the correlation between the gene expression profiles and P50 to a power β and modulating $\beta > 1$ did not affect the final gene list. For the identification of the DT module, transcripts were selected whose levels increased 3-fold between desiccation-sensitive and -tolerant stages, being at 11 and 23 DAP for high temperature, 16 and 32 DAP for greenhouse, 12 and 25 DAP for osmotic stress, 22 and 58 DAP for low temperature, and 14 and 32 DAP for standard conditions, respectively.

RT-qPCR Analysis

Reverse transcription reactions were performed on 1 μg of total RNA of 15 DAP seeds of Col-0, *wrky3*, and *nfxl1* lines using the QuantiTect Reverse Transcription Kit (Qiagen). Quantification of transcript levels of *WRKY33* (At2g38470), *HSPRO2* (At2g40000), *NFXL1* (At1g10170), and *LZF1* (At1g78600) were performed by RT-qPCR using an ABI PRISM 7100 sequence detection system and SYBR Green as a double-stranded DNA-specific fluorescent dye, using primers presented in Supplemental Table 4. Values are based on three repetitions. *TIP41-like* (At4g34270), *UBC21* (At5g25760), and *AP2M* (At5g46630) were used as reference genes.

Sugar Determination

Soluble sugar analysis on mature Arabidopsis seeds from the different lines was performed by Dionex using a Carbopac PA-1 column as previously described (Rosnoblet et al., 2007). Three biological replicates of 100 seeds that were harvested from three to six different plants were analyzed.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *WRKY33* (At2g38470), *HSPRO2* (At2g40000), *NFXL1* (At1g10170), *LZF1* (At1g78600), *TIP41-like* (At4g34270), *UBC21* (At5g25760), *AP2M* (At5g46630), *CAF-1* (At2g32070), *NOT4* (At3g45630), *AGAL2* (At5g08370), *SIP2* (At3g57520), *CYP81D3* (At4g37340), *PPC6-1* (At3g02750), *WRKY3* (At2g03340), *bHLH25* (At4g37850), and *FIP1* (At3g66652). Please refer to additional accession numbers in Supplemental Table 3.

Supplemental Data

Supplemental Figure 1. Characterization of network properties related to MatNet.

Supplemental Figure 2. Longevity of developing Arabidopsis seeds.

Supplemental Figure 3. Survival curves of wild-type and T-DNA mutant seeds of Arabidopsis during storage at 35°C and 75% RH.

Supplemental Figure 4. Absolute transcript levels of *NFLX1* and *WRKY3* in Arabidopsis development

Supplemental Figure 5. Dry weight and sugar content in mature seeds of *wkry3* and *nfxl1* mutants.

Supplemental Figure 6. Soluble sugar content in mature seeds of Arabidopsis wild type Col-0, *agal2*, and *sip2* mutants.

Supplemental Table 1. Insertion mutants (SALK, GK lines) of Arabidopsis used for the cross-species network validation.

Supplemental Table 2. Soluble sugar content in mature seeds of Col-0, *nfxl1*, and *wrky3* mutants.

Supplemental Table 3. Primers used for T-DNA insertion screening of Arabidopsis mutants.

Supplemental Table 4. Primers used for qPCR.

Supplemental Data Set 1. Genes present in MatNet

Supplemental Data Set 2. List of transcriptional regulators present in MatNet

Supplemental Data Set 3. Genes correlating with longevity acquisition in all environmental conditions and present in MatNet.

Supplemental Data Set 4. Genes correlating with desiccation tolerance acquisition in all environmental conditions and those present in MatNet.

Supplemental Data Set 5. Regulation of genes present in the desiccation tolerance and longevity module by ABI3.

Supplemental Data Set 6. Cross-species network of Arabidopsis and *Medicago truncatula* genes linked to longevity.

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AUTHOR CONTRIBUTIONS

K.R. performed research, analyzed the data, and wrote the article. J.L.V., B.L.V., J.V., and D.L. performed research. S.P., A.P., R.V.P., and N.J.P. analyzed data. J.B. and O.L. designed research, analyzed the data, and wrote the article.

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REFERENCES

Almoguera, C., Prieto-Dapena, P., Díaz-Martín, J., Espinosa, J.M., Carranco, R., and Jordano, J. (2009). The HaDREB2 transcription factor enhances basal thermotolerance and longevity of seeds through functional interaction with HaHSFA9. BMC Plant Biol. **9:** 75.

Amrine, K.C., Blanco-Ulate, B., and Cantu, D. (2015). Discovery of core biotic stress responsive genes in Arabidopsis by weighted gene co-expression network analysis. PLoS One **10:** e0118731.

Asano, T., Masuda, D., Yasuda, M., Nakashita, H., Kudo, T., Kimura, M., Yamaguchi, K., and Nishiuchi, T. (2008). AtNFXL1, an Arabidopsis homologue of the human transcription factor NF-X1, functions as a negative regulator of the trichothecene phytotoxin-induced defense response. Plant J. **53:** 450–464.

Baena-González, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature **448:** 938–942.

Bak, S., Tax, F.E., Feldmann, K.A., Galbraith, D.W., and Feyereisen, R. (2001). CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in Arabidopsis. Plant Cell **13:** 101–111.

Barabasi, A.L., and Albert, R. (1999). Emergence of scaling in random networks. Science **286:** 509–512.

Bassel, G.W., Lan, H., Glaab, E., Gibbs, D.J., Gerjets, T., Krasnogor, N., Bonner, A.J., Holdsworth, M.J., and Provart, N.J. (2011). Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. Proc. Natl. Acad. Sci. USA **108:** 9709–9714.

Baud, S., Boutin, J.P., Miquel, M., Lepiniec, L., and Rochat, C. (2002). An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiol. Biochem. **40:** 151–160.

Bentsink, L., Alonso-Blanco, C., Vreugdenhil, D., Tesnier, K., Groot, S.P., and Koornneef, M. (2000). Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of Arabidopsis. Plant Physiol. **124:** 1595–1604.

Bolingue, W., Rosnoblet, C., Leprince, O., Vu, B.L., Aubry, C., and Buitink, J. (2010). The MtSNF4b subunit of the sucrose non-fermenting-related kinase complex connects after-ripening and constitutive defense responses in seeds of *Medicago truncatula.* Plant J. **61:** 792–803.

Braybrook, S.A., Stone, S.L., Park, S., Bui, A.Q., Le, B.H., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2006). Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. Proc. Natl. Acad. Sci. USA **103:** 3468–3473.

Buitink, J., Hemminga, M.A., and Hoekstra, F.A. (2000). Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability. Plant Physiol. **122:** 1217–1224.

Buitink, J., and Leprince, O. (2010). Desiccation tolerance: From genomics to the field. Plant Sci. **179:** 554–564.

Carranco, R., Espinosa, J.M., Prieto-Dapena, P., Almoguera, C., and Jordano, J.R. (2010). Repression by an auxin/indole acetic acid protein connects auxin signaling with heat shock factor-mediated seed longevity. Proc. Natl. Acad. Sci. USA **107:** 21908–21913.

Chatelain, E., Hundertmark, M., Leprince, O., Le Gall, S., Satour, P., Deligny-Penninck, S., Rogniaux, H., and Buitink, J. (2012). Temporal profiling of the heat-stable proteome during late maturation of *Medicago truncatula* seeds identifies a restricted subset of late embryogenesis abundant proteins associated with longevity. Plant Cell Environ. **35:** 1440–1455.

Clerkx, E.J.M., Blankestijn-De Vries, H., Ruys, G.J., Groot, S.P.C., and Koornneef, M. (2004). Genetic differences in seed longevity of various Arabidopsis mutants. Physiol. Plant. **121:** 448–461.

Debeaujon, I., Léon-Kloosterziel, K.M., and Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. Plant Physiol. **122:** 403–414.

Delahaie, J., Hundertmark, M., Bove, J., Leprince, O., Rogniaux, H., and Buitink, J. (2013). LEA polypeptide profiling of recalcitrant and orthodox legume seeds reveals ABI3-regulated LEA protein abundance linked to desiccation tolerance. J. Exp. Bot. **64:** 4559–4573.

Dixon, R.A. (2005). Engineering of plant natural product pathways. Curr. Opin. Plant Biol. **8:** 329–336.

Faccioli, P., Provero, P., Herrmann, C., Stanca, A.M., Morcia, C., and Terzi, V. (2005). From single genes to co-expression networks: extracting knowledge from barley functional genomics. Plant Mol. Biol. **58:** 739–750.

Ficklin, S.P., Luo, F., and Feltus, F.A. (2010). The association of multiple interacting genes with specific phenotypes in rice using gene coexpression networks. Plant Physiol. **154:** 13–24.

Gallardo, K., Firnhaber, C., Zuber, H., Hericher, D., Belghazi, M., Henry, C., Kuster, H., and Thompson, R.D. (2007). A combined proteome and transcriptome analysis of developing. *Medicago truncatula* seeds. Mol. Cell. Proteomics **6:** 2165–2179.

Gissot, L., Polge, C., Jossier, M., Girin, T., Bouly, J.P., Kreis, M., and Thomas, M. (2006). AKINbetagamma contributes to SnRK1 heterotrimeric complexes and interacts with two proteins implicated in plant pathogen resistance through its KIS/GBD sequence. Plant Physiol. **142:** 931–944.

Glaab, E., Garibaldi, J.M., and Krasnogor, N. (2009). ArrayMining: a modular web-application for microarray analysis combining ensemble and consensus methods with cross-study normalization. BMC Bioinformatics **10:** 358.

He, J., Benedito, V.A., Wang, M., Murray, J.D., Zhao, P.X., Tang, Y., and Udvardi, M.K. (2009). The *Medicago truncatula* gene expression atlas web server. BMC Bioinformatics **10:** 441.

Horvath, S., and Dong, J. (2008). Geometric interpretation of gene coexpression network analysis. PLOS Comput. Biol. **4:** e1000117.

Hundertmark, M., Dimova, R., Lengefeld, J., Seckler, R., and Hincha, D.K. (2011). The intrinsically disordered late embryogenesis abundant

protein LEA18 from *Arabidopsis thaliana* modulates membrane stability through binding and folding. Biochim. Biophys. Acta **1808:** 446–453.

Junker, A., et al. (2012). Elongation-related functions of LEAFY COTYLEDON1 during the development of *Arabidopsis thaliana.* Plant J. **71:** 427–442.

Kim, J.I., Dolan, W.L., Anderson, N.A., and Chapple, C. (2015). Indole glucosinolate biosynthesis limits phenylpropanoid accumulation in *Arabidopsis thaliana.* Plant Cell **27:** 1529–1546.

Kranner, I., Birtić, S., Anderson, K.M., and Pritchard, H.W. (2006). Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? Free Radic. Biol. Med. **40:** 2155–2165.

Lai, Z., Vinod, K., Zheng, Z., Fan, B., and Chen, Z. (2008). Roles of Arabidopsis WRKY3 and WRKY4 transcription factors in plant responses to pathogens. BMC Plant Biol. **8:** 68.

Lee, T.-H., Kim, Y.-K., Pham, T.T.M., Song, S.I., Kim, J.-K., Kang, K.Y., An, G., Jung, K.-H., Galbraith, D.W., Kim, M., Yoon, U.-H., and Nahm, B.H. (2009). RiceArrayNet: a database for correlating gene expression from transcriptome profiling, and its application to the analysis of coexpressed genes in rice. Plant Physiol. **151:** 16–33.

Leubner-Metzger, G. (2003). Functions and regulation of b-1, 3-glucanases during seed germination, dormancy release and after-ripening. Seed Sci. Res. **13:** 17–34.

Li, H., Wang, L., and Yang, Z.M. (2015). Co-expression analysis reveals a group of genes potentially involved in regulation of plant response to iron-deficiency. Gene **554:** 16–24.

Lisso, J., Altmann, T., and Müssig, C. (2006). The *AtNFXL1* gene encodes a NF-X1 type zinc finger protein required for growth under salt stress. FEBS Lett. **580:** 4851–4856.

Liu, F., Jiang, H., Ye, S., Chen, W.P., Liang, W., Xu, Y., Sun, B., Sun, J., Wang, Q., Cohen, J.D., and Li, C. (2010). The Arabidopsis P450 protein CYP82C2 modulates jasmonate-induced root growth inhibition, defense gene expression and indole glucosinolate biosynthesis. Cell Res. **20:** 539–552.

Long, T.A., Brady, S.M., and Benfey, P.N. (2008). Systems approaches to identifying gene regulatory networks in plants. Annu. Rev. Cell Dev. Biol. **24:** 81–103.

Mönke, G., Seifert, M., Keilwagen, J., Mohr, M., Grosse, I., Hähnel, U., Junker, A., Weisshaar, B., Conrad, U., Bäumlein, H., and Altschmied, L. (2012). Toward the identification and regulation of the Arabidopsis thaliana ABI3 regulon. Nucleic Acids Res. **17:** 8240–8254.

Murray, S.L., Ingle, R.A., Petersen, L.N., and Denby, K.J. (2007). Basal resistance against *Pseudomonas syringae* in Arabidopsis involves WRKY53 and a protein with homology to a nematode resistance protein. Mol. Plant Microbe Interact. **20:** 1431–1438.

Mutwil, M., Usadel, B., Schütte, M., Loraine, A., Ebenhöh, O., and Persson, S. (2010). Assembly of an interactive correlation network for the Arabidopsis genome using a novel heuristic clustering algorithm. Plant Physiol. **152:** 29–43.

Nagel, M., Kranner, I., Neumann, K., Rolletschek, H., Seal, C.E., Colville, L., Fernández-Marín, B., and Börner, A. (2015). Genome-wide association mapping and biochemical markers reveal that seed ageing and longevity are intricately affected by genetic background and developmental and environmental conditions in barley. Plant Cell Environ. **38:** 1011–1022.

Nguyen, T.-P., Keizer, P., van Eeuwijk, F., Smeekens, S., and Bentsink, L. (2012). Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis. Plant Physiol. **160:** 2083–2092.

Ooms, J., Leon-Kloosterziel, K.M., Bartels, D., Koornneef, M., and Karssen, C.M. (1993). Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana* (A comparative study using abscisic acid-insensitive *abi3* mutants). Plant Physiol. **102:** 1185–1191.

Patel, R.V., Nahal, H.K., Breit, R., and Provart, N.J. (2012). BAR expressolog identification: expression profile similarity ranking of homologous genes in plant species. Plant J. **71:** 1038–1050.

Prieto-Dapena, P., Castaño, R., Almoguera, C., and Jordano, J. (2006). Improved resistance to controlled deterioration in transgenic seeds. Plant Physiol. **142:** 1102–1112.

Probert, R.J., Daws, M.I., and Hay, F.R. (2009). Ecological correlates of ex situ seed longevity: a comparative study on 195 species. Ann. Bot. (Lond.) **104:** 57–69.

Raiola, A., Lionetti, V., Elmaghraby, I., Immerzeel, P., Mellerowicz, E.J., Salvi, G., Cervone, F., and Bellincampi, D. (2011). Pectin methylesterase is induced in Arabidopsis upon infection and is necessary for a successful colonization by necrotrophic pathogens. Mol. Plant Microbe Interact. **24:** 432–440.

Rosnoblet, C., Aubry, C., Leprince, O., Vu, B.L., Rogniaux, H., and Buitink, J. (2007). The regulatory gamma subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. Plant J. **51:** 47–59.

Santos-Mendoza, M., Dubreucq, B., Baud, S., Parcy, F., Caboche, M., and Lepiniec, L. (2008). Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. Plant J. **54:** 608–620.

Sattler, S.E., Gilliland, L.U., Magallanes-Lundback, M., Pollard, M., and DellaPenna, D. (2004). Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. Plant Cell **16:** 1419–1432.

Schaefer, R.J., Briskine, R., Springer, N.M., and Myers, C.L. (2014). Discovering functional modules across diverse maize transcriptomes using COB, the Co-expression Browser. PLoS One **9:** e99193.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., and Lohmann, J.U. (2005). A gene expression map of *Arabidopsis thaliana* development. Nat. Genet. **37:** 501–506.

Schuck, S., Baldwin, I.T., and Bonaventure, G. (2013). HSPRO acts via SnRK1-mediated signaling in the regulation of Nicotiana attenuata seedling growth promoted by *Piriformospora indica.* Plant Signal. Behav. **8:** e23537.

Schwachtje, J., Minchin, P.E., Jahnke, S., van Dongen, J.T., Schittko, U., and Baldwin, I.T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proc. Natl. Acad. Sci. USA **103:** 12935–12940.

Song, Z., Krishna, S., Thanos, D., Strominger, J.L., and Ono, S.J. (1994). A novel cysteine-rich sequence-specific DNA-binding protein interacts with the conserved X-box motif of the human major histocompatibility complex class II genes via a repeated Cys-His domain and functions as a transcriptional repressor. J. Exp. Med. **180:** 1763–1774.

Sugliani, M., Rajjou, L., Clerkx, E.J.M., Koornneef, M., and Soppe, W.J.J. (2009). Natural modifiers of seed longevity in the Arabidopsis mutants *abscisic acid insensitive3-5* (*abi3-5*) and *leafy cotyledon1-3* (*lec1-3*). New Phytol. **184:** 898–908.

Terrasson, E., Darrasse, A., Righetti, K., Buitink, J., Lalanne, D., Ly Vu, B., Pelletier, S., Bolingue, W., Jacques, M.-A., and Leprince, O. (2015). Identification of a molecular dialogue between developing seeds of *Medicago truncatula* and seedborne xanthomonads. J. Exp. Bot. **66:** 3737–3752.

Usadel, B., Obayashi, T., Mutwil, M., Giorgi, F.M., Bassel, G.W., Tanimoto, M., Chow, A., Steinhauser, D., Persson, S., and

Provart, N.J. (2009). Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. Plant Cell Environ. **32:** 1633–1651.

Vandepoele, K., Quimbaya, M., Casneuf, T., De Veylder, L., and Van de Peer, Y. (2009). Unraveling transcriptional control in Arabidopsis using cis-regulatory elements and coexpression networks. Plant Physiol. **150:** 535–546.

Verdier, J., et al. (2013). A regulatory network-based approach dissects late maturation processes related to the acquisition of desiccation tolerance and longevity of *Medicago truncatula* seeds. Plant Physiol. **163:** 757–774.

Walters, D. (2010). Plant Defense: Warding Off Attack by Pathogens, Herbivores and Parasitic Plants. (Hoboken, NJ: Wiley-Blackwell).

Zhang, B., and Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. **4:** Article17.

Zheng, Z., Qamar, S.A., Chen, Z., and Mengiste, T. (2006). Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. **48:** 592–605.