the plant journal

SEB Society for Experimental Biology

The Plant Journal (2016) **85,** 451–465 doi: 10.1111/tpj.13118

The Arabidopsis *DELAY OF GERMINATION 1* gene affects *ABSCISIC ACID INSENSITIVE 5 (ABI5)* expression and genetically interacts with *ABI3* during Arabidopsis seed development

Bas J.W. Dekkers[1,2,3,†,*], Hanzi He[1,2,†], Johannes Hanson[3,4], Leo A.J. Willems[1,2], Diaan C.L. Jamar[2], Gwendal Cueff[5,6], Loïc Rajjou[5,6], Henk W.M. Hilhorst[1,2] and Leónie Bentsink[1,2]

[1]*Wageningen Seed Laboratory, Wageningen University, Droevendaalsesteeg 1, NL-6708 PB Wageningen, The Netherlands,*
[2]*Laboratory of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, NL-6708 PB Wageningen, The Netherlands,*
[3]*Department of Molecular Plant Physiology, Utrecht University, NL-3584 CH Utrecht, The Netherlands,*
[4]*Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-90187 Umeå, Sweden,*
[5]*INRA, Institut Jean-Pierre Bourgin (IJPB), UMR 1318 INRA/AgroParisTech, ERL CNRS 3559, Université Paris-Saclay, 'Saclay Plant Sciences' – RD10, F-78026 Versailles, France, and*
[6]*Chair of Plant Physiology, AgroParisTech, 16 rue Claude Bernard, F-75231 Paris Cedex 05, France*

Received 6 November 2015; revised 22 December 2015; accepted 23 December 2015; published online 5 January 2016.
*For correspondence (e-mail bas.dekkers@wur.nl)
[†]These authors contributed equally to this work.

SUMMARY

The seed expressed gene *DELAY OF GERMINATION* (*DOG*) *1* is absolutely required for the induction of dormancy. Next to a non-dormant phenotype, the *dog1-1* mutant is also characterized by a reduced seed longevity suggesting that *DOG1* may affect additional seed processes as well. This aspect however, has been hardly studied and is poorly understood. To uncover additional roles of *DOG1* in seeds we performed a detailed analysis of the *dog1* mutant using both transcriptomics and metabolomics to investigate the molecular consequences of a dysfunctional *DOG1* gene. Further, we used a genetic approach taking advantage of the weak *aba insensitive (abi) 3-1* allele as a sensitized genetic background in a cross with *dog1-1*. *DOG1* affects the expression of hundreds of genes including *LATE EMBRYOGENESIS ABUNDANT* and *HEAT SHOCK PROTEIN* genes which are affected by *DOG1* partly via control of *ABI5* expression. Furthermore, the content of a subset of primary metabolites, which normally accumulate during seed maturation, was found to be affected in the *dog1-1* mutant. Surprisingly, the *abi3-1 dog1-1* double mutant produced green seeds which are highly ABA insensitive, phenocopying severe *abi3* mutants, indicating that *dog1-1* acts as an enhancer of the weak *abi3-1* allele and thus revealing a genetic interaction between both genes. Analysis of the *dog1* and *dog1 abi3* mutants revealed additional seed phenotypes and therefore we hypothesize that *DOG1* function is not limited to dormancy but that it is required for multiple aspects of seed maturation, in part by interfering with ABA signalling components.

Keywords: DELAY OF GERMINATION, dormancy, seed development, seed maturation, ABA insensitive, abscisic acid, *Arabidopsis thaliana* (Arabidopsis).

INTRODUCTION

Seeds are important in the plant life cycle as they represent the link between two successive generations. The formation of seeds starts after fertilization and consists of two main phases, i.e. embryogenesis and maturation. During embryogenesis, here described for Arabidopsis (*Arabidopsis thaliana*), the embryo is formed via a well organized series of cell divisions and cell specification (Moller and Weijers, 2009) which is accomplished at the torpedo stage [around 7 days after pollination (DAP)] (Baud *et al.*, 2002). From 5 until 13 DAP the embryos go through a transient phase in which they become photosynthetically active (Allorent *et al.*, 2015). One of the proposed functions of this photosynthetic phase is to provide oxygen for seed respiration (Borisjuk and Rolletschek, 2009; Galili *et al.*,

2014). Chlorophyll breakdown (de-greening) is an important step during seed development as the retention of chlorophyll affects the storability of seeds in a negative way (Nakajima *et al.*, 2012). The beginning of the seed maturation phase is marked by a period of cell elongation triggering embryo growth which coincides with the start of storage compound accumulation (Baud *et al.*, 2002). By 10 DAP the embryo reaches the mature cotyledon stage and is now nearly fully grown in size although its dry weight increases due to continuous deposition of storage compounds. Around this stage seeds acquire the ability to germinate (Koornneef *et al.*, 1989), although this is rapidly suppressed by the establishment of seed dormancy (Raz *et al.*, 2001) which is defined as a temporary failure of a viable seed to complete germination under favourable conditions (Bewley, 1997). Dormancy is an important adaptive trait that allows more time for dispersal of seeds over larger distances and helps to time plant growth and reproduction in the most optimal conditions. Furthermore, orthodox seeds acquire desiccation tolerance (DT) (Koornneef *et al.*, 1989; Chatelain *et al.*, 2012; Dekkers *et al.*, 2015) that allows them to withstand extreme drying at the end of seed development. After the induction of DT, seed storability in the dry state is acquired during the later stages of seed maturation (Chatelain *et al.*, 2012; Righetti *et al.*, 2015). This is linked to the accumulation of raffinose family of oligosaccharides (RFO), increasing amounts of antioxidants, repair mechanisms for DNA and protein, seed storage proteins, as well as the expression of protectants like LATE EMBRYOGENESIS ABUNDANT (LEA) and HEAT SHOCK PROTEINs (HSP) (Wehmeyer and Vierling, 2000; Baud *et al.*, 2002; Bailly, 2004; Kotak *et al.*, 2007; Chatelain *et al.*, 2012; Rajjou *et al.*, 2012; Nguyen *et al.*, 2015). Seed desiccation finalizes the seed developmental program resulting in a mature dry seed around 20 DAP.

Four transcriptional regulators, *LEAFY COTYLEDON* (*LEC*) *1*, *LEC2*, *FUSCA* (*FUS*) *3* and *ABA INSENSITIVE* (*ABI*) *3/VIVIPAROUS (VP) 1* are indispensable for a proper maturation phase (McCarty *et al.*, 1989; Giraudat *et al.*, 1992; Lotan *et al.*, 1998; Luerssen *et al.*, 1998; Stone *et al.*, 2001; Santos-Mendoza *et al.*, 2008). For example, severe *abi3* mutants (like *abi3-5* and *abi3-6*) fail to accumulate seed storage proteins and RFOs, are badly storable and show a green seed phenotype due to the lack of chlorophyll degradation (Nambara *et al.*, 1992, 1995; Ooms *et al.*, 1993; Sugliani *et al.*, 2009; Delmas *et al.*, 2013). The plant hormone abscisic acid (ABA) is important for several aspects of maturation, including seed storage accumulation, DT and chlorophyll degradation (Phillips *et al.*, 1997; Finkelstein, 2013) and is critical for the induction of dormancy (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.*, 2008). Mutants that lack the capacity to produce ABA or transduce the ABA signal (e.g. *abi1-1*, *abi2-1* and *abi3-1*) are completely non-dormant (Koornneef *et al.*, 1982, 1984). Conversely, mutants that over-accumulate ABA or those that show an ABA hypersensitive phenotype have enhanced dormancy levels (Cutler *et al.*, 1996; Kushiro *et al.*, 2004). ABA-responsive transcription factors *ABI4* and *ABI5* are seemingly not involved in the induction of primary dormancy (Finkelstein, 1994) although a weak dormancy phenotype has been reported for the *abi4* mutant (Shu *et al.*, 2013). However, both transcription factors may contribute to the induction of DT (Maia *et al.*, 2014) and do regulate the expression of seed maturation-related genes (Finkelstein *et al.*, 1998; Finkelstein and Lynch, 2000; Nakabayashi *et al.*, 2005; Reeves *et al.*, 2011). For example, ABI5 binds the ABA RESPONSE ELEMENT (ABRE) present in the *EARLY METHIONINE-LABELLED* (*EM) 1*/*LEA1* and *EM6*/*LEA6* promoters to regulate their expression (Bensmihen *et al.*, 2002; Carles *et al.*, 2002; Reeves *et al.*, 2011).

Screening of natural variation for seed dormancy in Arabidopsis, led to the identification of *DELAY OF GERMINATION* (*DOG*) *1* (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006). *DOG1* encodes a protein of unknown function that is, like ABA, critical for the induction of seed dormancy and both regulators were suggested to act in largely parallel pathways to regulate dormancy (Nakabayashi *et al.*, 2012). *DOG1* expression is regulated by environmental signals (mainly temperature) during dormancy cycling of seeds in the soil or during development on the mother plant and the level of DOG1 protein is well correlated with the depth of seed dormancy (Footitt *et al.*, 2011; Kendall *et al.*, 2011; Nakabayashi *et al.*, 2012; Graeber *et al.*, 2014). Most interestingly, it was shown that during after-ripening the DOG1 protein is modified which potentially reduces its activity and therefore it was suggested that DOG1 protein may act as a timer for dormancy release (Nakabayashi *et al.*, 2012). Despite the pivotal role of *DOG1* in the induction of dormancy, its mode of action and full function are still unclear. For example, *dog1* mutants show a reduced longevity (Bentsink *et al.*, 2006) indicating that *DOG1* has other functions beyond dormancy during seed maturation however this aspect has hardly been investigated. We used a combination of 'omics technologies, a genetics approach and physiological experimentation to gain a further understanding of the function of *DOG1* during seed maturation. This work reveals that *DOG1*, next to dormancy, affects multiple aspects of seed maturation in part by interfering with components in ABA signalling.

RESULTS

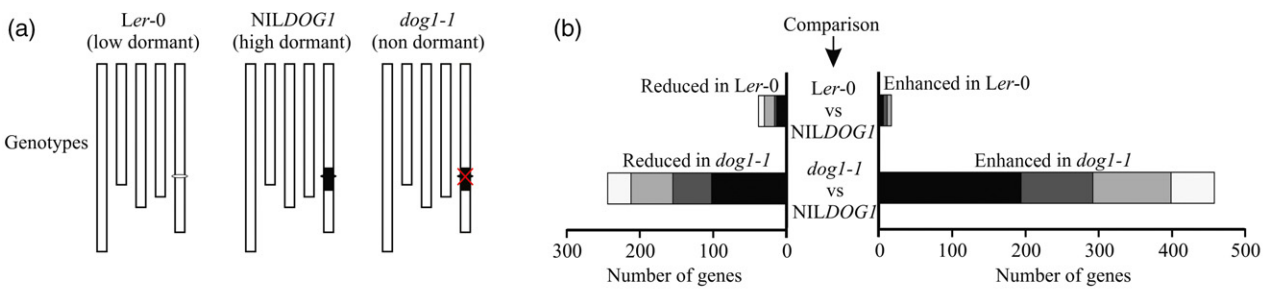
The dry seed transcriptome is severely affected in *dog1-1*

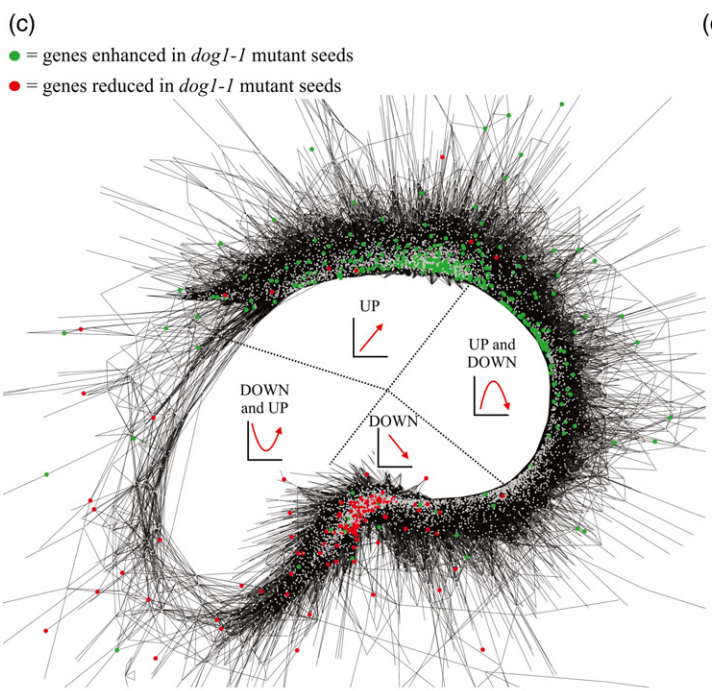
The DOG1 protein accumulates from 14 DAP and remains present at a steady level throughout the later stages of seed maturation till the mature dry seed (Nakabayashi *et al.*, 2012). In order to investigate the regulatory function of *DOG1* we have performed transcriptome analyses using

dry seeds (the end product of seed maturation) of three genotypes with different *DOG1* expression levels including L*er*-0 WT, the near isogenic line NIL*DOG1*-Cvi and the non-dormant *dog1-1* mutant. NIL*DOG1*-Cvi is the L*er*-0 WT containing an introgression of the Cvi accession on chromosome 5, which includes the *DOG1* gene (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006) (Figure 1a). It has a strong expression of the *DOG1* gene in comparison with L*er*-0 (Nakabayashi *et al.*, 2012). The *dog1-1* mutant is generated in the NIL*DOG1* background (Figure 1a) and has a one base pair deletion resulting in a lack of any detectable DOG1 protein accumulation and is considered to be a full knock-out (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012).

Comparing the dry seed transcriptome of L*er*-0 (low *DOG1* expression) with NIL*DOG1* (high *DOG1* expression) revealed a low number of differentially expressed genes [39 genes down- and 17 genes up-regulated in L*er*-0 (>2-fold; $P < 0.05$)]. In *dog1-1* seeds 458 genes were up- and 245 down-regulated as compared with NIL*DOG1* seeds (Figure 1b and Data S1). Thus the lack of *DOG1* has a profound effect on the dry seed transcriptome.

We plotted the 703 differentially expressed genes in *dog1-1* in the co-expression network, EndoNet (Figure 1c). This co-expression network is inferred from gene transcript expression information of endosperm samples that were collected in a dense time series encompassing the





(d)

Exemplar overrepresented functional classes found in the set of 458 genes with an enhanced expression in *dog1-1* mutant dry seeds.

Functional class	Genes found	Genes expected	*P*-value
Growth	19	6.8	4.9e-4
Plant-type cell wall loosening	5	0.7	1.9e-3
Cell wall organization or biogenesis	13	5.5	1.4e-2
Cell morphogenesis	13	5.1	8.8e-3
Ribosome biogenesis	20	3.0	6.8e-10
Translation	47	10.5	3.5e-16
Protein metabolic process	85	54.6	1.8e-4
Carbohydrate metabolic process	33	16.8	1.1e-6
Response to sucrose stimulus	6	0.7	4.9e-4
Organic acid metabolic process	24	13.9	2.2e-2
Gene expression	67	48.2	1.2e-2
Response to stimulus	141	70.3	1.4e-15
Response to gibberellin stimulus	11	2.5	4.3e-4
Response to ethylene stimulus	8	2.9	2.9e-2
Response to jasmonic acid stimulus	10	3.2	7.6e-3
Response to stress	92	40.9	2.6e-12
Response to temperature stimulus	25	8.0	7.6e-6
Response to oxidative stress	14	5.6	8.0e-3
Response to osmotic stress	36	8.4	6.8e-12
Water channel activity	7	0.6	2.6e-5

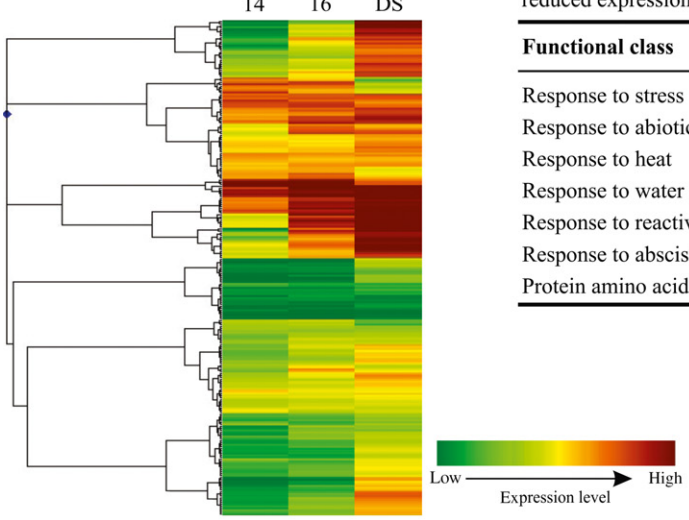
Figure 1. Transcriptome analysis of *dog1-1* mutant seeds. (a) Graphical representation of the three genotypes that were used for the transcriptome study (L*er*-0, NIL*DOG1* and *dog1-1*) each having with a different primary dormancy level. NIL*DOG1* has a L*er*-0 genetic background with an introgression of Cvi (indicated in black) surrounding the *DOG1* gene (indicated by a bar). The *dog1-1* mutant is generated in the NIL*DOG1* background. (b) The graph depicts the number of genes that are differentially expressed (>2-fold; $P < 0.05$) between the L*er*-0 and NIL*DOG1* and NIL*DOG1* and *dog1-1* mutant. Different fold change cut-offs are indicated by colour, black >2-fold, dark grey >2.5-fold, light grey >3-fold and white >5-fold. (c) The genes that are either enhanced or reduced in *dog1-1* (in comparison to NIL*DOG1*) are plotted in EndoNet co-expression network encompassing seed germination (Dekkers *et al.*, 2013). In the middle of the network the four dominant expression profiles during Arabidopsis seed germination are indicated. (d) Exemplar functional classes found by overrepresentation analysis of genes with enhanced expression in *dog1-1* using Genetrail.

germination time course from dry seeds to radicle protrusion of Arabidopsis seeds (Dekkers *et al.*, 2013). The gene sets that are either up- or down-regulated in *dog1-1* are mostly separated and positioned differently in this network. The down-regulated gene set is positioned in a region which is characterized by genes that are down-regulated during germination while the majority of the genes that are up-regulated in *dog1-1* dry seeds are located in the region of the network that consists of genes that are induced during germination (Figure 1c). This set consisted of over 10% of genes related to translation and other functional classes that are overrepresented included those related to growth, cell wall modification, response to gibberellin, carbohydrate metabolic process and response to stress (Figure 1d and Data S1). This finding suggests that *DOG1* functions as an important repressor of germination-expressed genes.

The expression of genes related to late seed maturation and desiccation is impaired in *dog1-1* seeds

Next, we focussed on the 245 genes that are down-regulated in *dog1-1*. We used a microarray dataset encompassing late maturation and seed desiccation consisting of Arabidopsis seeds sampled at 14 days after pollination (DAP), 16 DAP and mature dry seeds (Angelovici *et al.*, 2009) to visualize the gene expression patterns of this set of genes during seed maturation. Many of these genes increased between 14 DAP and the dry seed stage in this data set (Figure 2a), indicating that many genes that are induced during late seed maturation fail to do so in *dog1-1*. This set was overrepresented for functional classes which are predominantly related to ABA and stress (Figure 2b and Data S1). It included 10 genes related to ABA signalling (Table S1) and 27 genes (of 245; 11%) that were

(a)

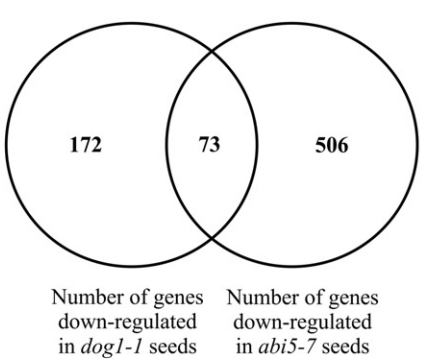


(b)

Exemplar overrepresented functional classes found in the set of 245 genes with a reduced expression in *dog1-1* mutant dry seeds.

Functional class	Genes found	Genes expected	*P*-value
Response to stress	47	21.760	1.31e-5
Response to abiotic stimulus	38	13.506	4.43e-7
Response to heat	22	1.542	6.84e-17
Response to water deprivation	7	2.144	3.69e-2
Response to reactive oxygen species	9	0.759	2.71e-6
Response to abscisic acid stimulus	13	3.168	4.99e-4
Protein amino acid dephosphorylation	5	0.686	7.59e-3

(c)



(d)

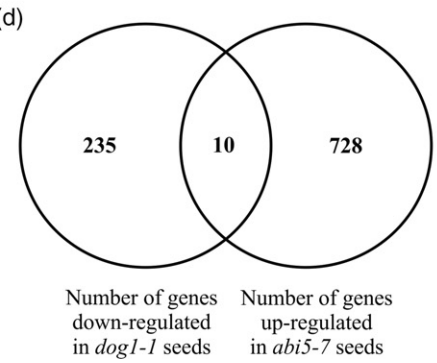


Figure 2. A set of 245 genes is differentially down-regulated in *dog1-1* seeds. (a) Expression profiles of the 245 genes that are expressed at a lower level in *dog1-1* seeds at three time points during seed development (14, 16 DAP and dry seeds). This shows that the expression of many genes in this set is increased during the last stages of seed development. We used a data set that has been published previously by Angelovici *et al.* (2009). The colour key is shown indicating low expression in green and high expression in red. (b) Exemplar functional classes found by overrepresentation analysis of the down-regulated genes in *dog1-1* using Genetrail. (c) Venn diagram showing the overlap of genes down-regulated in both *dog1-1* and *abi5-7* mutant seeds. (d) As a control we also investigated the overlap between the down-regulated set in *dog1-1* with the up-regulated set in *abi5-7*.

classified as either *LEA* or *HEAT SHOCK PROTEIN* (*HSP*) (Table S2). A striking observation is that the ABA set contained the bZIP transcription factor *ABI5*, a homologous bZIP transcription factor gene *ABRE-BINDING FACTOR* (*ABF*) *4* (also known as *ABA RESPONSE ELEMENT BINDING 2* (Uno *et al.*, 2000)) and four *ABI FIVE BINDING PROTEINS (AFP) 1-4* (Table S1). We confirmed the down-regulation of both *ABI5* and *ABF4* transcription factor genes by RT-qPCR in two *dog1* alleles (Figure S1).

ABI5 transcript abundance increases late during seed maturation and peaks the last 2 days of seed development and Nakabayashi *et al.* (2005) identified over 500 genes that were down-regulated in dry seeds of the *abi5-7* mutant. We identified a large overlap of 73 genes (out of 245 *dog1-1* down-regulated genes, 29.8%) with genes which are down-regulated in *abi5* (Figure 2c,d). This situation opens up the possibility that *DOG1* induces the expression of two ABA signalling-related transcription factors, *ABI5* and *ABF4*, which in turn regulate downstream gene expression and this idea was explored further using a genetic approach. To investigate the role of *ABF4* and possible redundancy with *ABI5* we isolated two homozygous *ABF4* T-DNA knock-out lines, SALK_069523/*abf4-1* and SALK_043475/*abf4-2* (Figure 3a,b). We investigated the expression of 13 *dog1-1* down-regulated genes including six *LEA*, three *HSP*s, one transcription factor and three genes with unknown function (Table S3) by RT-qPCR in *abf4-1* and *abf4-2*. However, none of the investigated genes was significantly affected in expression level consistently in both of the mutant alleles, indicating that *ABF4* is not essential for the regulation of their gene expression (Figure 3c).

Next, we investigated the expression of the same 13 genes in dry seeds of six genotypes; Col-0 WT, the *abi5-7* mutant (Nambara *et al.*, 2002) which has an early stop codon in its sequence (Tamura *et al.*, 2006), the *dog1-4* mutant (Bentsink *et al.*, 2006) which has an transposon insertion in the first exon, the double mutant *abi5-7 dog1-4*, the double mutant *abi5-7 abf4-1* and the triple mutant *abi5-7 dog1-4 abf4-1* (Figure 3d–g). Four genes were significantly down-regulated in the *abi5-7* mutant (Figure 3e) while five other genes were significantly down-regulated in *abi5-7* and even significantly lower expressed in the *abi5-7 abf4-1* double mutant (Figure 3f). This result revealed gene redundancy between *ABI5* and *ABF4* and indicated that *ABF4* was also involved in their regulation. For seven out of these nine investigated genes, the expression value of the *abi5-7 dog1-4* double mutant equalled that of one of the single mutants, indicating that *DOG1* and *ABI5* act in a linear genetic pathway which may include *ABF4*. All genes were down-regulated in the *dog1-4* mutant (Figure 3e–g) confirming the expression analysis of the *dog1-1* mutant. The expression was often more severely reduced in the *dog1-4* single mutant compared with the *abi5-7* or a reduced expression of the triple mutant compared with the *abi5-7 abf4-1* double mutant, indicating that other factors than *ABI5*/*ABF4* act downstream of *DOG1* to regulate the expression of these genes as well (Figure 3h).

The content of specific amino acids, N-rich compounds, sugars and the TCA cycle intermediate fumarate is changed in *dog1-1* seeds

As the transcriptome analysis provided a strong indication that *dog1-1* seeds are affected in late seed maturation we investigated whether this effect was also reflected in its metabolome. Therefore, primary metabolites were extracted from mature dry seeds of L*er*-0, NIL*DOG1* and *dog1-1* and analysed by gas chromatography–time-of-flight mass spectrometry (GC–TOF MS). In total, 124 metabolites/centrotypes were detected of which 41 metabolites were identified. Compared with NIL*DOG1* the content of eight compounds was lower (fold change >1.5 and a $P < 0.05$) in *dog1-1* seeds (Figure 4a,b). The most severely affected metabolites were asparagine (Asn; 80-fold down), aspartate (Asp; 51-fold down), allantoin (22-fold down) and urea (17-fold down). Other metabolites with lower levels in *dog1-1* were phenylalanine (Phe), fumarate, galactinol and raffinose. Four metabolites (*N*-acetylglutamic acid (NAc-Glu); serine (Ser); 1,6-anhydroglucose and ethanolamine) were significantly increased in the *dog1-1* mutant but the fold changes were generally smaller.

We investigated the changes in sugar content in more detail and more accurately by high pressure liquid chromatography (HPLC) analysis in *dog1-1*, and seeds of two other non-dormant genotypes, i.e. *abi3-1* and *aba2-1*. The monosaccharides glucose, fructose and xylose accumulated to higher levels in the *dog1-1* mutant but to lower levels (glucose and xylose) in *aba2-1* (Figure 5a–c). Sucrose levels were significantly enhanced in *abi3-1* and *aba2-1* but were unaffected in *dog1-1* (Figure 5d) The RFO pathway (see Figure 5e) has been implicated in stress resistance and seed storability (Obendorf, 1997; Nishizawa *et al.*, 2008). In *dog1-1* the whole pathway from galactinol to stachyose is down-regulated in dry seeds (Figure 5e–h). In *abi3-1* the levels of stachyose were lower but surprisingly the level of raffinose was significantly enhanced suggesting that the conversion from raffinose to stachyose is blocked in this genotype (Figure 5g,h). In the *aba2-1* mutant galactinol content was significantly lower but the level of raffinose was enhanced and that of stachyose was unaffected compared with the WT (Figure 5f–h). This result indicates that *DOG1* negatively affects the accumulation of the monosaccharides glucose, fructose and xylose but that a functional *DOG1* is required for a proper accumulation of RFO pathway compounds galactinol, raffinose and stachyose. The differential accumulation of these sugars in the three non-dormant genotypes indicated that the changes in *dog1-1* were not a secondary effect of being non-dormant.

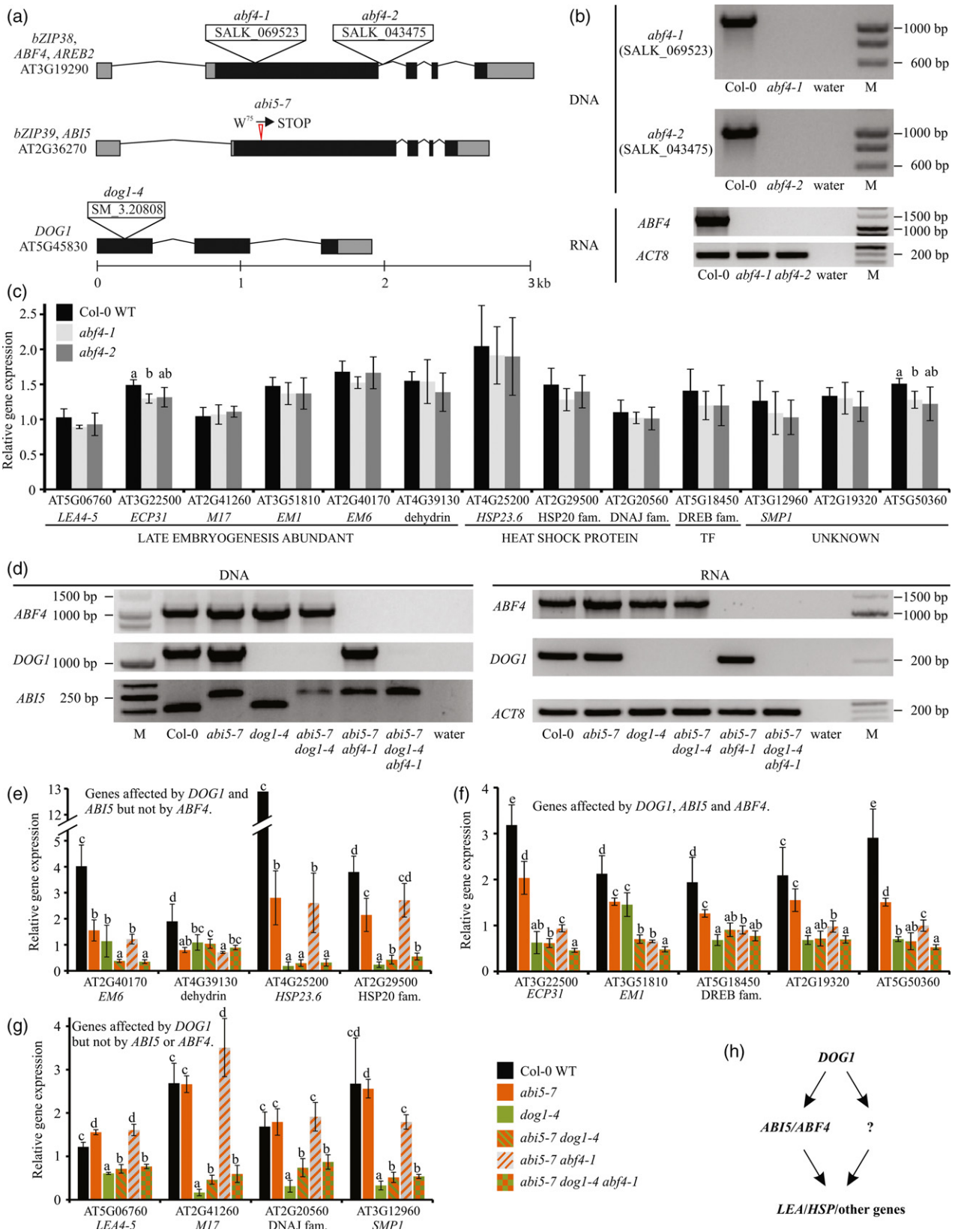


Figure 3. *DOG1* and *ABI5* acts in a genetic pathway to regulate downstream gene expression. (a) The gene models of *ABF4, ABI5* and *DOG1* are shown indicating the sites of and nature of the mutations in the mutants used. (b) Genotyping of two *ABF4* SALK T-DNA insertion lines. Two primer pairs each flanking one of the insertions sites amplified the predicted fragment in Col-0 WT but not in the homozygous T-DNA mutants. The T-DNA insertions in *ABF4* were confirmed on the RNA level using primers flanking both insertion sites, as only in the Col-0 WT the expected 1265-bp fragment was amplified using amplification of *ACT8* as a control. (c) The gene expression levels of 13 genes (which were found to be down-regulated in the *dog1-1* mutant) in *abf4-1* and *abf4-2*. The values are means ± standard deviation (SD) of four biological replicates. Letters indicate a significant difference between the genotypes (using Student's *t*-test, $P < 0.05$). For the genes where no letters are indicated there were no significant differences detected between the three genotypes. (d) Genotyping of Col-0 WT, the single mutants *abi5-7* and *dog1-4*, the double mutants *abi5-7 dog1-4* and *abi5-7 abf4-1* and the triple mutant *abi-5-7 dog1-4 abf4-1*. Genotyping for *ABF4* and *DOG1* was done by primers flanking the T-DNA insertion sites while for ABI5 a dCAPS marker was developed to detect the single nucleotide polymorphism (SNP) in the *abi5-7* mutant. The insertions in *ABF4* and *DOG1* were confirmed on the RNA level using amplification of *ACT8* as a control. (e–g) The expression levels of 13 genes are shown, including six *LEA*, three *HSP*, one transcription factor (TF) and three genes of unknown function in Col-0, *abi5-7, dog1-4, abi5-7 dog1-4, abi5-7 abf4-1* and *abi5-7 dog1-4 abf4-1*. The values are means ± standard deviation (SD) of five biological replicates. Letters indicate a significant difference between the genotypes (using Student's *t*-test, $P < 0.05$). (e) Four genes were down-regulated in *dog1-4* and *abi5-7*, although no additional reduction in expression was observed in the *abi5-7 abf4-1* mutant. (f) Five genes were down-regulated in *dog1-4* and *abi5-7* and the expression was further reduced in the *abi5-7 abf4-1* compared with the *abi5-7* single mutant. (g) Four genes were down-regulated in *dog1-4* but in neither the *abi5-7* nor the *abi5-7 abf4-1* double mutant. (h) The genetic analysis supports a model in which *DOG1* affects downstream gene expression, in part, by positively affecting *ABI5* and *ABF4* gene expression.

The *dog1-1* mutant is an enhancer of the weak *abi3-1* allele

ABI3 encodes a B3 domain-containing transcriptional regulator (McCarty *et al.*, 1989; Giraudat *et al.*, 1992) and is an important regulator of seed maturation. Severe mutations in this gene (such as *abi3-5*) show distorted seed development: mature seeds are green, non-dormant, impaired in storage protein accumulation, and have a severely compromised longevity (Ooms *et al.*, 1993; Nambara *et al.*, 1995). In contrast, the *abi3-1* (a weak allele) mutant seeds are ABA insensitive and non-dormant but their development is relatively normal. Seeds are brown (Figure 6a), desiccation tolerant and storable for considerable time (Ooms *et al.*, 1993; Clerkx *et al.*, 2004). We employed the *abi3-1* mutant as a sensitized genetic background in a cross with *dog1-1*. Surprisingly, the seeds of the *dog1-1 abi3-1* double mutant showed the green seed phenotype, a resultant from a lack of chlorophyll degradation during maturation (Figure 6a). The double mutant *dog1-1 abi3-1* as well as the severe *abi3-5* mutant showed the highest chlorophyll fluorescence, as expected (Figure 6b). Although not visible by eye, compared with wild type the *abi3-1* seeds also had slightly elevated chlorophyll levels based on the fluorescence measurements, which is in agreement with previous chlorophyll measurements (Clerkx *et al.*, 2003). Moreover, these seeds displayed a strongly reduced longevity upon harvest (Figure 6c,d). Similar to the severe *abi3-5* mutant, the *dog1-1 abi3-1* double mutant is highly insensitive to ABA (Figure 6d). Interestingly, at higher concentrations of ABA, seeds of the double mutant germinate (i.e. show radicle protrusion) but do not establish seedlings in contrast to the strong *abi3-5* mutant (Figure 6d). Sugar analysis revealed higher levels of mono- and disaccharides (glucose, fructose, xylose, sucrose, maltose) and a severely reduced accumulation of RFOs in *dog1-1 abi3-1*, similar to the *abi3-5* mutant (Figure S2).

To further substantiate these observations the proteome of five genotypes (L*er*-0, *dog1-1*, *abi3-1*, *dog1-1 abi3-1* and *abi3-5*) was analysed by LC-MS/MS. This method enabled us to detect 473 proteins. A principal component analysis (PCA) was used to analyse the proteome dataset and to obtain an insight in the grouping of the different samples. Based on the protein profiles, L*er*-0 and *dog1-1* clustered closely together (Figure 6e). On the other side of the plot, the double mutant *dog1-1 abi3-1* and *abi3-5* clustered in close proximity as well, indicating that they appeared similar based on their protein profiles in agreement with the other phenotypes. The single *abi3-1* mutant was positioned between the other four genotypes, suggesting an intermediate phenotype (Figure 6e). The green seed phenotype, higher chlorophyll fluorescence, reduced storability and ABA insensitivity were reminiscent of severe *abi3* mutants (Ooms *et al.*, 1993; Nambara *et al.*, 1995; Sugliani *et al.*, 2009), indicating that *dog1-1* acts as an enhancer of the weak *abi3-1* mutation. Seed storage proteins CRUCIFERIN (CRU) 2, CRU3; ALBUMIN 1, 4 and 5; and OLEOSIN proteins (that function as structural components of oil bodies) were not significantly changed in the *dog1-1* mutant compared with WT but were found to be severely reduced in *abi3-1*, *abi3-5* and the double mutant (Figure S3). Thus, several phenotypes, including ABA sensitivity, chlorophyll degradation and seed storage protein accumulation, were not affected in the single *dog1-1* mutant but could only be revealed using *abi3-1* as a sensitized genetic background (Figures 6 and 7).

DISCUSSION

DOG1 controls multiple aspects of the seed maturation program

Several agricultural problems relate directly to a suboptimal seed maturation phase. For example, a low level or a lack of dormancy may result, under cool and moist conditions, in germination of seeds that are still attached to the mother plant (known as pre-harvest sprouting or vivipary) (Gubler *et al.*, 2005). Similarly, a reduced chlorophyll degradation negatively affects seed storability and, in oil seeds, the quality of the oil extracted from such seeds (Johnson-Flanagan *et al.*, 1994; Clerkx *et al.*, 2003;

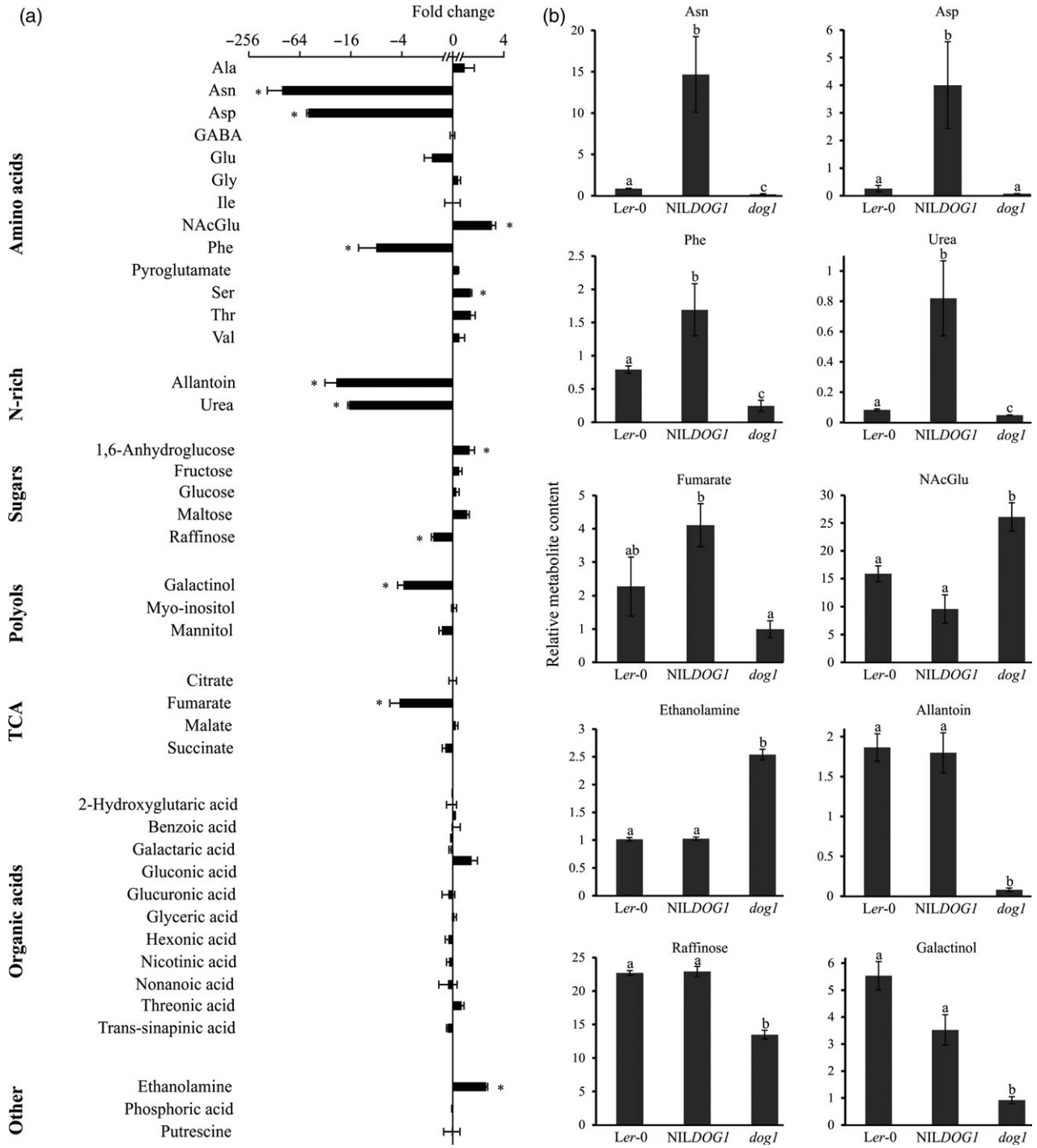


Figure 4. Primary metabolite content of *dog1-1* seeds. (a) The graph shows the accumulation of the 41 identified primary metabolites in *dog1-1* relative to NIL-*DOG1*. Asterisks indicate significant fold change (using Student's *t*-test, $P < 0.05$ and a fold change difference of >1.5). (b) Shows the relative amounts of 10 individual metabolites (Asn, Asp, Phe, NAcGlu, allantoin, urea, raffinose, galactinol, fumarate, ethanolamine) in all three genotypes. Values represent means ± standard error of the mean (SEM) of three biological replicates indicating the response of the metabolite, normalized to the internal standard ribitol as well as to the mean of the entire sample set for each metabolite. Letters indicate a significant difference between the genotypes (using Student's *t*-test, $P < 0.05$).

Nakajima *et al.*, 2012). Such issues result in a low quality end product and cause significant economic losses (Whitmarsh and Ortiz-Lopez, 2000; Gubler *et al.*, 2005; Dekkers and Bentsink, 2015) indicating the need for an improved understanding of this important developmental phase. Detailed analyses of the *dog1* mutant presented in this

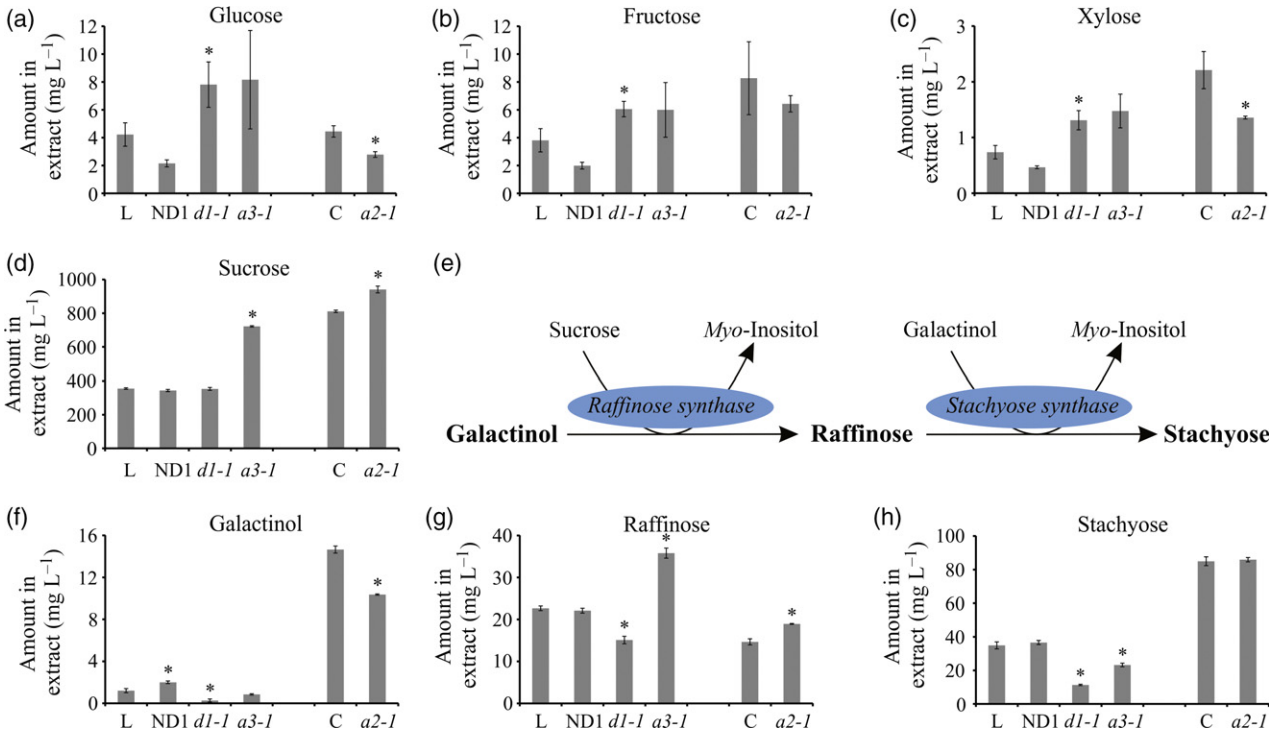


Figure 5. Sugar content in dry seeds of three non-dormant genotypes. (a–d) The graphs show the sugar contents of the monosaccharides (a) glucose, (b) fructose, (c) xylose and (d) the disaccharide sucrose. (e) Overview of the biosynthetic pathway of RFOs, from its precursor galactinol. (f–h) The graphs below show levels of (f) galactinol, (g) raffinose, and (h) stachyose measured in extracts of 10 mg of dry seeds. The values are means ± standard error of the mean (SEM) of three biological replicates. Genotypes are indicated as L = L*er*-0, ND1 = NIL*DOG1*, *d1-1* = *dog1-1*, *a3-1* = *abi3-1*, C = Col-0, *a2-1* = *aba2-1*. Significant differences (using Student's *t*-test, $P < 0.05$) are indicated by '*' above the bars of NIL*DOG1*, *dog1-1*, *abi3-1* and *aba2-1* in comparison with their respective WTs.

work showed that it is affected in many aspects of seed maturation (Figure 7) and leads us to hypothesize that *DOG1* is a regulator of seed maturation. As *ABI5* and *ABF4* expression (among others) is reduced in the *dog1-1* transcriptome and *DOG1* and *ABI3* genetically interact, as revealed from the *dog1-1 abi3-1* double mutant analysis, suggested that *DOG1* does so, in part, by targeting components of the ABA signalling pathway. Moreover, LEC2 and FUS3 have been suggested to bind directly to the *DOG1* promoter (Braybrook *et al.*, 2006; Wang and Perry, 2013) indicating that *DOG1* is part of a genetic network in which it is tightly linked with core regulators of seed maturation. Interestingly, another well known positive regulator of ABA signalling, the AP2-type transcription factor *ABI4* (Finkelstein *et al.*, 1998), showed an increased relative transcript abundance in dry *dog1* seeds (Data S1 and Figure S1). This result is in agreement with the observation that *ABI4* is induced during germination and its higher expression levels in *dog1* are indicative for its precocious activation as observed for many germination-induced genes (Figure 1).

The study of *dog1-1* seed transcriptome revealed a reduced expression of a substantial number of *HSP*s and *LEA*s, which act as molecular chaperones, enzyme protectants or antioxidants (Ellis and Vandervies, 1991; Tunnacliffe and Wise, 2007). Together with RFOs (whose accumulation is also disturbed in *dog1-1* seeds, Figure 5) they may function as 'fillers' to maintain cellular integrity (Hoekstra *et al.*, 2001; Farrant *et al.*, 2007). Additionally, in the proteome of *dog1-1* ChlADR (AT1G54870) is reduced (Figure S3a), this gene is implicated in detoxifying reactive carbonyls that are produced as a result of lipid peroxidation (Yamauchi *et al.*, 2011). Such changes in *dog1-1* may underly the reduced seed longevity phenotype (Figure 6c, d; Bentsink *et al.*, 2006).

ABI5 acts downstream of *DOG1* as a regulator of gene expression

We found a remarkable overlap in the genes down-regulated in the *abi5-7* mutant (Nakabayashi *et al.*, 2005) as compared with *dog1-1*, suggesting that the differences in the *dog1-1* transcriptome could, in part, be affected by the reduced expression of the *ABI5* transcription factor. We provided additional evidence for this using a genetic approach which showed that *DOG1* and *ABI5* act in a linear genetic pathway which however, also implicates the involvement of additional factors. A likely candidate being one of these additional factors is *HEAT SHOCK FACTOR* (*HSF*) *A9.* This factor encodes a transcription factor that is expressed during the later stages of seed development

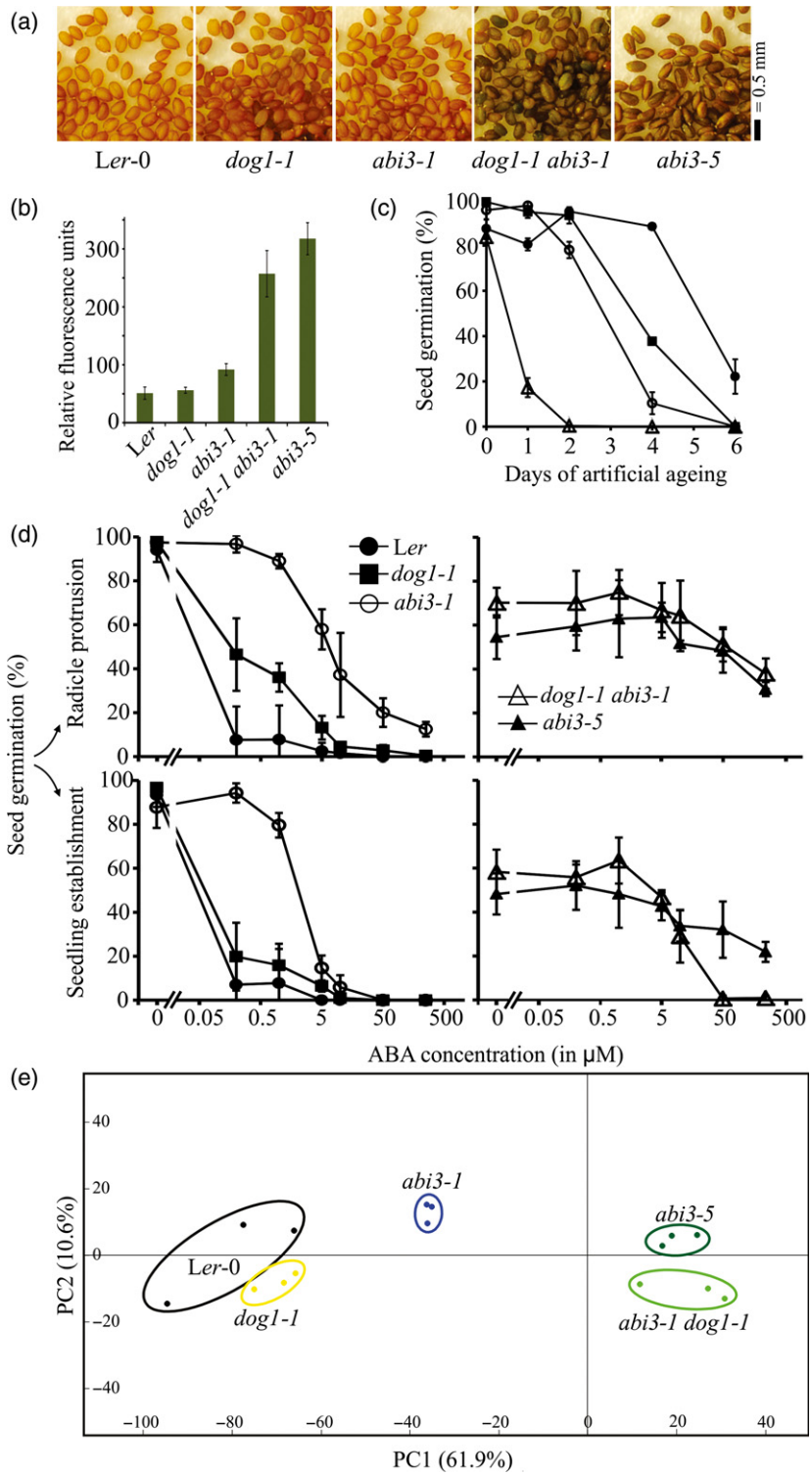


Figure 6. Seed related phenotypes of the *dog1-1 abi3-1* double mutant. (a) The *dog1-1 abi3-1* double mutant shows a green seed phenotype. Photographs show mature dry seeds of the different genotypes. (b) Chlorophyll fluorescence of seeds of the different genotypes as a measure of chlorophyll content. (c) The line graph shows the effect of an artificial ageing treatment on L*er*-0, *dog1-1*, *abi3-1* and the double mutant *dog1-1 abi3-1*. The same symbols are used to represent the genotypes as in panel (d). (d) ABA dose–response curve. Germination was measured by both radicle protrusion and seedling establishment. The data of *abi3-5* and *dog1-1 abi3-1* are plotted in a separated graph. (e) The individuals samples of the proteome analysis of L*er*-0, *dog1-1*, *abi3-1*, *abi3-5* and the double mutant *dog1-1 abi3-1* are plotted in a PCA plot.

(Kotak *et al.*, 2007). These authors proposed a transcriptional cascade in which ABI3 activates, either directly or indirectly, the expression of *HSFA9*, which in turn binds to the promoters of three *HSP* genes (*HSP17.4* AT3G46230; *HSP17.6A*, AT5G12030; *HSP101*, AT1G74310) to induce their expression. *HSFA9* and all three targets were down-regulated in the *dog1-1* mutant suggesting that *DOG1* activates this transcriptional cascade as well. It is unlikely that this process occurs via *ABI3* mRNA expression as this is unaffected in the *dog1-1* mutant.

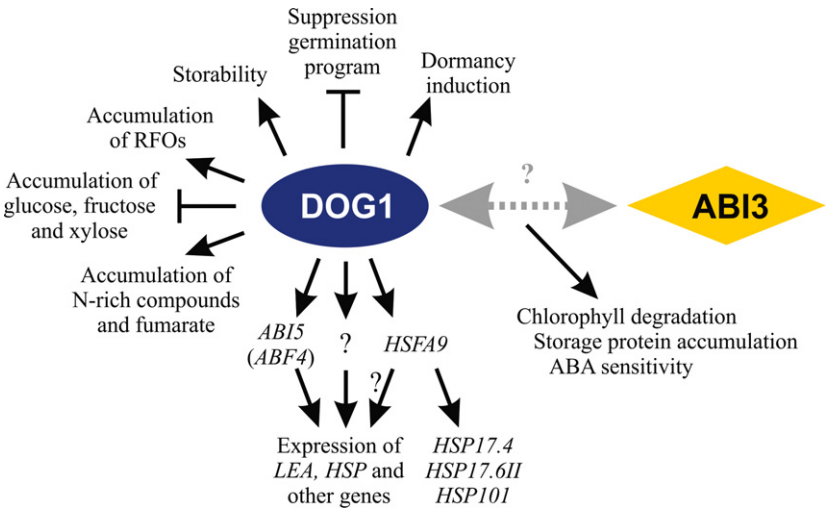


Figure 7. *DOG1* affects multiple aspects of seed maturation. The figure summarizes the different functions that emerged by detailed analysis of the *dog1-1* mutant and the possible role of several TFs that regulate a part of the transcriptome downstream of *DOG1*. Some of the functions of *DOG1* were only identified using a sensitized genetic background (*abi3-1*), which suggest that these are regulated in conjunction with *ABI3*, although the nature of their interaction is as yet unknown.

ABI5 belongs to the 13-member group A of Arabidopsis bZIP transcription factors (Jakoby *et al.*, 2002). It has been shown that members within this group can act both redundantly (Finkelstein *et al.*, 2005; Yoshida *et al.*, 2010) or antagonistically (Bensmihen *et al.*, 2002). ABF4 was identified as an ABA response element binding protein (Choi *et al.*, 2000; Uno *et al.*, 2000) which mediates ABA signalling (Kang *et al.*, 2002) and is part of the same bZIP transcription factor group as ABI5. Our data showed that *ABF4* is involved in the expression of a subset of the investigated genes as well, although *ABF4* proved not to be essential. This finding is similar to results reported by Finkelstein *et al.* (2005) in which they identified the bZIP transcription factor *ABF3* as acting redundantly to *ABI5.* Also *ABF3* was not essential for *EM1* and *EM6* expression (Finkelstein *et al.*, 2005).

Most of the genes that are differentially expressed in *dog1-1* seeds are enhanced in expression. Many of these up-regulated genes are activated during germination and this set is overrepresented for gene categories supporting germination. This finding suggests that an important function of *DOG1* is to repress genes related to germination, which is in agreement with its function in dormancy control. We found an overlap of 92 genes (out of 458 genes, 20%) with genes enhanced in the *abi5-7* mutant (Nakabayashi *et al.*, 2005) (Figure S4). Thus we find an large overlap between genes differentially expressed in dry seeds of *dog1-1* and *abi5-7*, suggesting that *DOG1* activates *ABI5*, not only to induce maturation genes but also to repress a set of germination related transcripts.

DOG1 affects contents of metabolites that change during the transition from seed maturation to seed desiccation

At the end of seed development the accumulation of storage lipids is halted and a small part is remobilized (Baud *et al.*, 2002; Chia *et al.*, 2005). Simultaneously, sugars (sucrose, galactinol, raffinose and stachyose), the TCA intermediates fumarate and succinate and free amino acids accumulate while hexoses decrease during the last stages of seed development (Baud *et al.*, 2002; Fait *et al.*, 2006). The phenotypes observed in *dog1-1* seeds suggest that *DOG1* modulates the decrease in hexoses and the increase of the compounds of the RFO pathway (Figures 5 and 7). With respect to the large changes on the metabolite level, the transition from seed maturation to seed desiccation is characterized by a metabolic switch (Fait *et al.*, 2006). A subset of the metabolites involved in this switch is affected in *dog1-1.* For example fumarate was found to increase during desiccation although the mechanism of its accumulation is unclear (Fait *et al.*, 2006). Our data open up the possibility that this metabolic shift is, in part, affected by *DOG1* activity.

The green seed phenotype of *dog1-1 abi3-1* seeds reveal a genetic interaction between *DOG1* and *ABI3*, a master regulator of seed maturation

In the cross between *dog1-1* and *abi3-1* we utilized the leaky nature of the *abi3-1* allele as a sensitized genetic background. Such a background is useful to identify genetic modifiers and enhancer mutations (those that aggravate the mutant phenotype) that are predicted to identify genes acting redundantly with the primary mutation (Page and Grossniklaus, 2002). Mutagenesis of *abi3-1* seeds identified several of such enhancers. Examples are *green seed* (*grs;* Clerkx *et al.*, 2003) as well as several intragenic enhancers including the severe *abi3-4* allele (Ooms *et al.*, 1993). The double mutant *dog1-1 abi3-1* also has green seeds showing that *dog1-1* is another, previously unknown, enhancer of *abi3-1. Dog1-1 abi3-1* differs from the *grs abi3-1* double mutant in that the *dog1-1 abi3-1* showed a strongly enhanced ABA insensitivity (Figure 6d). Two important questions emergence from these results.

The first is how *dog1-1* enhances the *abi3-1* phenotype. Reduced ABA levels (caused by the *aba1-1*-mutation) combined with the *abi3-1* mutation also produced green seeds (Koornneef *et al.*, 1989). Therefore, we cannot rule out an effect of the lower ABA levels observed in the *dog1-1* mutant (Nakabayashi *et al.*, 2012) to affect the *abi3-1* phenotype, among other possibilities. Secondly, what is exactly the genetic relationship between *DOG1* and *ABI3;* do they act in parallel or does one acts upstream of the other? Nakabayashi *et al.* (2012) postulated that *DOG1* and ABA act in largely parallel pathways to regulate dormancy, although it is assumed that they merge downstream. *ABI3* is an important regulator of ABA signalling making it a possible point of convergence between both pathways. Answers to these questions may provide important insights in DOG1 interactions and function.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of the *Arabidopsis thaliana* (L.) Heynh. accessions Columbia (Col-0) and Landsberg *erecta* (L*er*-0) were used in this study. L*er*-0, NIL*DOG1*, *dog1-1* and *dog1-4* were retrieved as described by Bentsink *et al.* (2006). The *dog1-3* (He *et al.*, 2014), *abf4-1* (SALK_069523) and *abf4-2* (SALK_043475) are SALK T-DNA insertion mutants (Alonso *et al.*, 2003) and were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Genotyping was performed by standard PCR using primers obtained via T-DNA primer design web page of the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html). Primers used to confirm the T-DNA insertion on the RNA level by PCR are shown in Table S4. The *abi5-7* mutant is described by Nambara *et al.* (2002) and genotyped using dCAPS marker developed using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) (Neff *et al.*, 2002). *Abi5-7* dCAPS marker: For: CGTCAGAGCGAGAAGTAGAG Rev: GCGGGGCGGGGGCACGGGGGGGATTGTTATTATTCTCCTCTGCGAT, restriction with *Dpn*II which digests the WT fragment. The isolations of *abi3-1* and *abi3-5* mutants are described by Koornneef *et al.* (1984) and Ooms *et al.* (1993), respectively. To obtain the double mutant *dog1-1 abi3-1* the F2 individuals of this cross were screened for their ability to form seedlings on ABA-containing media. In this way we selected for individuals homozygote for the *abi3-1* mutation. The ABA resistant individuals were than screened for the Cvi introgression surrounding the *DOG1* gene by PCR using marker K15I22 which is a single sequence length polymorphism (Bentsink *et al.*, 2006). The individuals that we identified as being both ABA resistant and homozygous for the introgression produced green seeds that were highly ABA insensitive (Figure 6). For the phenotypic, metabolome and proteome analyses plants were grown on 4 × 4 cm Rockwool blocks in a growth chamber at 20°C/18°C (day/night) under a 16-h photoperiod of artificial light (150 μmol m^{-2} s^{-1}) and 70% relative humidity. Plants were watered three times per week with a standard nutrient solution (He *et al.*, 2014).

Microarray analysis

For the transcriptome analyses L*er*-0, NIL*DOG1* and *dog1-1* were grown in a randomized complete block design with three replicates, each consisting of a bulk of eight plants. The growth conditions used were described (el-Lithy *et al.*, 2006). RNA was extracted from freshly harvested seeds of three biological replicates of each genotype for hybridization on Affymetrix ATH1 GeneChips. RNA extraction, quality assessment, processing and hybridization were according to Bentsink *et al.* (2010). The resulting data were analysed using the R statistical programming environment (R-Core-Team 2013) and the Bioconductor packages Limma (Gentleman *et al.*, 2004) and Affy (Gautier *et al.*, 2004). The raw data were normalized with Robust Microarray Averaging (RMA) (Irizarry *et al.*, 2003), using a custom chip definition file (.cdf) from the CustomCDF project (Ath1121501_At_TAIRG.cdf v18.0.0, released 23 January 2014 (Dai *et al.*, 2005) obtained via http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/18.0.0/tairg.asp. The microarray data were deposited in NCBI's Gene Expression Omnibus (GEO number GSE65471).

Significant differential expression changes were computed using the Limma package (Smyth, 2004) and *P*-values were adjusted for multiple testing with the Benjamini–Hochberg method to control for false positives (Benjamini and Hochberg, 1995). A gene was considered differentially expressed between two genotypes if the difference between mean signal of the genotypes was over two-fold and statistically significant (adjusted *P*-value of 0.05 or lower). Microarray quality and reproducibility data are presented in Figure S5. Overrepresentation analysis was performed using Genetrail (Keller *et al.*, 2008).

RT-qPCR analysis

For RT-qPCR, RNA was isolated from dry mature seeds using a phenol/chloroform extraction method (described in Schuurmans *et al.*, 2003) or a hot borate-based protocol (Maia *et al.*, 2011). Sample preparation, RNA quality assessment and primer design and testing have been described previously (Dekkers *et al.*, 2012, 2013). RNA (700 ng) was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Laboratories B.V., Veenendaal, The Netherlands, www.bio-rad.com). cDNA samples were diluted in a volume of 380 μL using sterile MilliQ water. qPCR reactions consisted of 3 μL sample, 6 μL iQ SYBR Green Supermix (Bio-Rad), 0.3 μL of primer (from a 10 μM work solution) and was supplemented with water to a final volume 12 μL. Three technical replicates were run per sample. The RT-qPCR reactions were run on a CFX machine (Bio-Rad). The RT-qPCR data were loaded in qbasePLUS (Hellemans *et al.*, 2007) (Biogazelle, Ghent, Belgium, www.biogazelle.com). For data normalization we ran four to seven reference genes that are stably expressed in seeds (Dekkers *et al.*, 2012). The two most stably expressed genes identified by the geNORM program (Vandesompele *et al.*, 2002), were used for normalization. Primers used for RT-qPCR are listed in Table S5.

Primary metabolite analysis by GC-TOF-MS

The metabolite extraction was performed on dry mature seeds of L*er*-0, NIL*DOG1* and *dog1-1* based on a previously described method (Roessner *et al.*, 2000) with some modifications. For each genotype metabolite extractions were performed on three biological replicates. Details of the metabolite extraction, GC-TOF-MS analysis and data processing are described in Methods S1.

Sugar measurements

Sugar contents were determined as described by Bentsink *et al.* (2000), with minor modifications. Ten mg of dry seeds were homogenized in 1 mL of methanol (80% v/v) with the addition of 20 μg of melezitose as internal standard. The homogenate was heated for 15 min at 76°C and centrifuged 5 min at 10 000 ***g***. The supernatant was vacuum evaporated, and its residue was resuspended in 0.5 mL of MilliQ water and injected into a Dionex

ICS5000$^+$ HPLC system with electrochemical detection. Sugars were chromatographed using a CarboPac PA1 4 × 250 mm column preceded by a guard column (CarboPac PA1 4 × 50 mm). Mono-, di-, and trisaccharides were separated by elution in an increasing concentration of NaOH (20–350 mM) with a flow rate of 1 mL/min. Peaks were identified by co-elution of standards. Sugar quantity was corrected using melezitose as an internal standard.

Phenotyping seed traits

Several phenotypic traits were assessed for the different genotypes, including L*er*-0, *dog1-1*, *abi3-1*, *abi3-5* and the double mutant *dog1-1 abi3-1.* Chlorophyll fluorescence was measured using a Junior pulse-amplitude modulated chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The measurements were performed on four biological replicates and values are relative fluorescence units. A controlled deterioration test was performed assays were performed to assess longevity of the genotypes under study. Seeds were treated at 40°C and a relative humidity of 80% for up to 6 days and survival was assessed by germination. The germination assays were performed as described by Joosen *et al.* (2010) using the Germinator set-up. Six samples, of approximately 50–100 seeds, were sown on two layers of blue germination papers equilibrated with 50 mL of demineralized water in plastic trays (15 × 21 cm). Trays were piled and wrapped in a closed and transparent plastic bag. The bags were incubated in an incubator at 22°C and continuous light. Germination was followed daily by taking photos. ABA sensitivity was assessed using a dose response curve using the amounts as indicated. We used the germination assay as above with the difference that in this case we assessed germination both by radicle protrusion as well as seedling establishment.

Total soluble protein extraction

In total, 15 mg of dry seeds of each sample (three biological replicates) were ground with mortar and pestle in liquid nitrogen for about 1 min. Extraction buffer and protease inhibitor, as previously described by Rajjou *et al.* (2008), were added into seed powder, followed by a 2-min grinding. The extract was recovered into 1.5 mL eppendorf tube and incubated with DNase I, RNase A, and DTT at 4°C for 1 h on a rotating disc. The total soluble protein extract was collected as supernatant after centrifugation with 20 000 ***g*** at 4°C for 10 min. Details of protein quantification and identification are described in Methods S1.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Organization for Scientific Research (NWO) (to BJWD and LB), by the Dutch Technology Foundation (STW), which is the applied science division of The Netherlands Organization for Scientific Research and the Technology Program of the Ministry of Economic Affairs (to HH and LAJW*)* and Bio4Energy, a Strategic Research Environment appointed by the Swedish government (to JH). We are grateful to Thierry Balliau from the PAPPSO platform (Gif/Yvette, France) for having performed mass spectrometry analysis.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Confirmation of *dog1-1* microarray data by RT-qPCR.

Figure S2. Seed sugar content of the *dog1-1 abi3-1* double mutant mimics that of the severe *abi3-5* mutant.

Figure S3. Proteome analysis of the *dog1* and *abi3* mutants.

Figure S4. Overlap between up-regulated gene sets in *abi5-7* and *dog1-1* seeds.

Figure S5. Microarray quality and reproducibility.

Table S1. List of genes related to ABA signalling that are down-regulated in *dog1-1* mutant seeds.

Table S2. Late embryogenesis abundant and heat shock protein genes that are down-regulated in *dog1-1.*

Table S3. Overview of 13 genes whose expression level was tested by RT-qPCR in *abf4*, *abi5*, *dog1*, *abi5 dog1*, *abi5 abf4* and *abi5 dog1 abf4* mutants.

Table S4. Primers sets used for genotyping *ABF4* and *DOG1* T-DNA insertion mutants on the mRNA level.

Table S5. Primer sets used for RT-qPCR.

Methods S1. Detailed description of the metabolite analysis by GC-TOF-MS and protein quantification and identification.

Data S1. Sets of differentially expressed genes in the *dog1-1* mutant seeds.

REFERENCES

Allorent, G., Osorio, S., Vu, J.L. *et al.* (2015) Adjustments of embryonic photosynthetic activity modulate seed fitness in *Arabidopsis thaliana*. *New Phytol.* **205**, 707–719.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., Blankestijn-de Vries, H. and Koornneef, M. (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics*, **164**, 711–729.

Angelovici, R., Fait, A., Zhu, X., Szymanski, J., Feldmesser, E., Fernie, A.R. and Galili, G. (2009) Deciphering transcriptional and metabolic networks associated with lysine metabolism during Arabidopsis seed development. *Plant Physiol.* **151**, 2058–2072.

Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* **14**, 93–107.

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. Bioch.* **40**, 151–160.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Statist. Soc. B. Mec.* **57**, 289–300.

Bensmihen, S., Rippa, S., Lambert, G., Jublot, D., Pautot, V., Granier, F., Giraudat, J. and Parcy, F. (2002) The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. *Plant Cell*, **14**, 1391–1403.

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.

Bentsink, L., Jowett, J., Hanhart, C.J. and Koornneef, M. (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **103**, 17042–17047.

Bentsink, L., Hanson, J., Hanhart, C.J. *et al.* (2010) Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. *Proc. Natl Acad. Sci. USA*, **107**, 4264–4269.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell*, **9**, 1055–1066.

Borisjuk, L. and Rolletschek, H. (2009) The oxygen status of the developing seed. *New Phytol.* **182**, 17–30.

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.

Carles, C., Bies-Etheve, N., Aspart, L., Leon-Kloosterziel, K.M., Koornneef, M., Echeverria, M. and Delseny, M. (2002) Regulation of *Arabidopsis thaliana Em* genes: role of ABI5. *Plant J.* **30**, 373–383.

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.

Chia, T.Y., Pike, M.J. and Rawsthorne, S. (2005) Storage oil breakdown during embryo development of *Brassica napus* (L.). *J. Exp. Bot.* **56**, 1285–1296.

Choi, H., Hong, J., Ha, J., Kang, J. and Kim, S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *J. Biol. Chem.* **275**, 1723–1730.

Clerkx, E.J., Vries, H.B., Ruys, G.J., Groot, S.P. and Koornneef, M. (2003) Characterization of green seed, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity. *Plant Physiol.* **132**, 1077–1084.

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.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. *Science*, **273**, 1239–1241.

Dai, M., Wang, P., Boyd, A.D. *et al.* (2005) Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res.* **33**, e175.

Dekkers, B.J.W. and Bentsink, L. (2015) Regulation of seed dormancy by abscisic acid and *DELAY OF GERMINATION 1*. *Seed Sci. Res.* **25**, 82–98.

Dekkers, B.J., Willems, L., Bassel, G.W., van Bolderen-Veldkamp, R.P., Ligterink, W., Hilhorst, H.W. and Bentsink, L. (2012) Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and tomato seeds. *Plant Cell Physiol.* **53**, 28–37.

Dekkers, B.J., Pearce, S., van Bolderen-Veldkamp, R.P. *et al.* (2013) Transcriptional dynamics of two seed compartments with opposing roles in Arabidopsis seed germination. *Plant Physiol.* **163**, 205–215.

Dekkers, B.J., Costa, M.C., Maia, J., Bentsink, L., Ligterink, W. and Hilhorst, H.W. (2015) Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance. *Planta*, **241**, 563–577.

Delmas, F., Sankaranarayanan, S., Deb, S., Widdup, E., Bournonville, C., Bollier, N., Northey, J.G.B., McCourt, P. and Samuel, M.A. (2013) ABI3 controls embryo degreening through Mendel's *I* locus. *Proc. Natl Acad. Sci. USA*, **110**, E3888–E3894.

Ellis, R.J. and Vandervies, S.M. (1991) Molecular Chaperones. *Annu. Rev. Biochem.* **60**, 321–347.

Fait, A., Angelovici, R., Less, H., Ohad, I., Urbanczyk-Wochniak, E., Fernie, A.R. and Galili, G. (2006) Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. *Plant Physiol.* **142**, 839–854.

Farrant, J.M., Brandt, W. and Lindsey, G.G. (2007) An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants. *Plant Stress*, **1**, 72–84.

Finch-Savage, W.E. and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol.* **171**, 501–523.

Finkelstein, R.R. (1994) Mutations at two new Arabidopsis ABA response loci are similar to the *abi3* mutations. *Plant J.* **5**, 765–771.

Finkelstein, R. (2013) Abscisic acid synthesis and response. *Arabidopsis Book*, **11**, e0166.

Finkelstein, R.R. and Lynch, T.J. (2000) The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. *Plant Cell*, **12**, 599–609.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S. and Goodman, H.M. (1998) The Arabidopsis abscisic acid response locus *ABI4* encodes an APETALA 2 domain protein. *Plant Cell*, **10**, 1043–1054.

Finkelstein, R., Gampala, S.S.L., Lynch, T.J., Thomas, T.L. and Rock, C.D. (2005) Redundant and distinct functions of the ABA response loci ABA-INSENSITIVE(ABI)5 and ABRE-BINDING FACTOR (ABF)3. *Plant Mol. Biol.* **59**, 253–267.

Finkelstein, R., Reeves, W., Ariizumi, T. and Steber, C. (2008) Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.* **59**, 387–415.

Footitt, S., Douterelo-Soler, I., Clay, H. and Finch-Savage, W.E. (2011) Dormancy cycling in Arabidopsis seeds is controlled by seasonally distinct hormone-signaling pathways. *Proc. Natl Acad. Sci. USA*, **108**, 20236–20241.

Galili, G., Avin-Wittenberg, T., Angelovici, R. and Fernie, A.R. (2014) The role of photosynthesis and amino acid metabolism in the energy status during seed development. *Front. Plant Sci.* **5**, 447.

Gautier, L., Cope, L., Bolstad, B.M. and Irizarry, R.A. (2004) affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*, **20**, 307–315.

Gentleman, R.C., Carey, V.J., Bates, D.M. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the Arabidopsis *ABI3* gene by positional cloning. *Plant Cell*, **4**, 1251–1261.

Graeber, K., Linkies, A., Steinbrecher, T. *et al.* (2014) DELAY OF GERMINATION 1 mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. *Proc. Natl Acad. Sci. USA*, **111**, E3571–E3580.

Gubler, F., Millar, A.A. and Jacobsen, J.V. (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr. Opin. Plant Biol.* **8**, 183–187.

He, H., de Souza Vidigal, D., Snoek, L.B., Schnabel, S., Nijveen, H., Hilhorst, H. and Bentsink, L. (2014) Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. *J. Exp. Bot.* **65**, 6603–6615.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **8**, R19.

Hoekstra, F.A., Golovina, E.A. and Buitink, J. (2001) Mechanisms of plant desiccation tolerance. *Trends Plant Sci.* **6**, 431–438.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. and Speed, T.P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, **4**, 249–264.

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. and Parcy, F. and **bZIP Research Group**. (2002) bZIP transcription factors in Arabidopsis. *Trends Plant Sci.* **7**, 106–111.

Johnson-Flanagan, A.M., Maret, L.L.D. and Pomeroy, M.K. (1994) Humidification of green canola seed leads to pigment degradation in the absence of germination. *Crop Sci.* **34**, 6.

Joosen, R.V., Kodde, J., Willems, L.A., Ligterink, W., van der Plas, L.H. and Hilhorst, H.W. (2010) GERMINATOR: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. *Plant J.* **62**, 148–159.

Kang, J.Y., Choi, H.I., Im, M.Y. and Kim, S.Y. (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell*, **14**, 343–357.

Keller, A., Backes, C., Al-Awadhi, M., Gerasch, A., Kuntzer, J., Kohlbacher, O., Kaufmann, M. and Lenhof, H.P. (2008) GeneTrailExpress: a web-based pipeline for the statistical evaluation of microarray experiments. *BMC Bioinformatics*, **9**, 552.

Kendall, S.L., Hellwege, A., Marriot, P., Whalley, C., Graham, I.A. and Penfield, S. (2011) Induction of dormancy in Arabidopsis summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors. *Plant Cell*, **23**, 2568–2580.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L. and Karssen, C.M. (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) heynh. *Theor. Appl. Genet.* **61**, 385–393.

Koornneef, M., Reuling, G. and Karssen, C.M. (1984) The isolation and characterization of abscisic-acid insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **61**, 377–383.

Koornneef, M., Hanhart, C.J., Hilhorst, H.W. and Karssen, C.M. (1989) In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiol.* **90**, 463–469.

Kotak, S., Vierling, E., Baumlein, H. and von Koskull-Doring, P. (2007) A novel transcriptional cascade regulating expression of heat stress proteins during seed development of Arabidopsis. *Plant Cell*, **19**, 182–195.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y. and Nambara, E. (2004) The Arabidopsis cytochrome P450CYP707A encodes ABA 8 '-hydroxylases: key enzymes in ABA catabolism. *EMBO J.* **23**, 1647–1656.

el-Lithy, M.E., Bentsink, L., Hanhart, C.J., Ruys, G.J., Rovito, D., Broekhof, J.L., van der Poel, H.J., van Eijk, M.J., Vreugdenhil, D. and Koornneef, M. (2006) New Arabidopsis recombinant inbred line populations genotyped using SNPWave and their use for mapping flowering-time quantitative trait loci. *Genetics*, **172**, 1867–1876.

Lotan, T., Ohto, M., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (1998) Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, **93**, 1195–1205.

Luerssen, H., Kirik, V., Herrmann, P. and Misera, S. (1998) FUSCA3 encodes a protein with a conserved VP1/AB13-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. *Plant J.* **15**, 755–764.

Maia, J., Dekkers, B.J., Provart, N.J., Ligterink, W. and Hilhorst, H.W. (2011) The re-establishment of desiccation tolerance in germinated *Arabidopsis thaliana* seeds and its associated transcriptome. *PLoS ONE*, **6**, e29123.

Maia, J., Dekkers, B.J., Dolle, M.J., Ligterink, W. and Hilhorst, H.W. (2014) Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated Arabidopsis seeds. *New Phytol.* **203**, 81–93.

McCarty, D.R., Carson, C.B., Stinard, P.S. and Robertson, D.S. (1989) Molecular analysis of viviparous-1 - an abscisic acid-insensitive mutant of maize. *Plant Cell*, **1**, 523–532.

Moller, B. and Weijers, D. (2009) Auxin control of embryo patterning. *Cold Spring Harb. Perspect. Biol.* **1**, a001545.

Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y. and Nambara, E. (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant J.* **41**, 697–709.

Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R., Seo, M. and Soppe, W.J.J. (2012) The time required for dormancy release in Arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *Plant Cell*, **24**, 2826–2838.

Nakajima, S., Ito, H., Tanaka, R. and Tanaka, A. (2012) Chlorophyll b reductase plays an essential role in maturation and storability of Arabidopsis seeds. *Plant Physiol.* **160**, 261–273.

Nambara, E., Naito, S. and McCourt, P. (1992) A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new *Abi3* allele. *Plant J.* **2**, 435–441.

Nambara, E., Keith, K., McCourt, P. and Naito, S. (1995) A regulatory role for the *Abi3* Gene in the establishment of embryo maturation in *Arabidopsis thaliana*. *Development*, **121**, 629–636.

Nambara, E., Suzuki, M., Abrams, S., McCarty, D.R., Kamiya, Y. and McCourt, P. (2002) A screen for genes that function in abscisic acid signaling in *Arabidopsis thaliana*. *Genetics*, **161**, 1247–1255.

Neff, M.M., Turk, E. and Kalishman, M. (2002) Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet.* **18**, 613–615.

Nguyen, T.P., Cueff, G., Hegedus, D.D., Rajjou, L. and Bentsink, L. (2015) A role for seed storage proteins in Arabidopsis seed longevity. *J. Exp. Bot.* **66**, 6399–6413.

Nishizawa, A., Yabuta, Y. and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.* **147**, 1251–1263.

Obendorf, R.L. (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. *Seed Sci. Res.* **7**, 63–74.

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.

Page, D.R. and Grossniklaus, U. (2002) The art and design of genetic screens: *Arabidopsis thaliana*. *Nat. Rev. Genet.* **3**, 124–136.

Phillips, J., Artsaenko, O., Fiedler, U., Horstmann, C., Mock, H.P., Muntz, K. and Conrad, U. (1997) Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. *EMBO J.* **16**, 4489–4496.

Rajjou, L., Lovigny, Y., Groot, S.P., Belghazi, M., Job, C. and Job, D. (2008) Proteome-wide characterization of seed aging in Arabidopsis: a comparison between artificial and natural aging protocols. *Plant Physiol.* **148**, 620–641.

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C. and Job, D. (2012) Seed germination and vigor. *Annu. Rev. Plant Biol.* **63**, 507–533.

Raz, V., Bergervoet, J.H. and Koornneef, M. (2001) Sequential steps for developmental arrest in Arabidopsis seeds. *Development*, **128**, 243–252.

R-Core-Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Reeves, W.M., Lynch, T.J., Mobin, R. and Finkelstein, R.R. (2011) Direct targets of the transcription factors ABA-Insensitive(ABI)4 and ABI5 reveal synergistic action by ABI4 and several bZIP ABA response factors. *Plant Mol. Biol.* **75**, 347–363.

Righetti, K., Vu, J.L., Pelletier, S. *et al.* (2015) Inference of longevity-related genes from a robust coexpression network of seed maturation identifies regulators linking seed storability to biotic defense-related pathways. *Plant Cell* **10**, 2692–2708.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N. and Willmitzer, L. (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J.* **23**, 131–142.

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.

Schuurmans, J.A., van Dongen, J.T., Rutjens, B.P., Boonman, A., Pieterse, C.M. and Borstlap, A.C. (2003) Members of the aquaporin family in the developing pea seed coat include representatives of the PIP, TIP, and NIP subfamilies. *Plant Mol. Biol.* **53**, 633–645.

Shu, K., Zhang, H., Wang, S., Chen, M., Wu, Y., Tang, S., Liu, C., Feng, Y., Cao, X. and Xie, Q. (2013) ABI4 regulates primary dormancy by regulating the biogenesis of abscisic acid and gibberellins in Arabidopsis. *PLoS Genet.* **9**, e1003577.

Smyth, G.K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**. Article3.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (2001) LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proc. Natl Acad. Sci. USA*, **98**, 11806–11811.

Sugliani, M., Rajjou, L., Clerkx, E.J., Koornneef, M. and Soppe, W.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.

Tamura, N., Yoshida, T., Tanaka, A., Sasaki, R., Bando, A., Toh, S., Lepiniec, L. and Kawakami, N. (2006) Isolation and characterization of high temperature-resistant germination mutants of Arabidopsis thaliana. *Plant Cell Physiol.* **47**, 1081–1094.

Tunnacliffe, A. and Wise, M.J. (2007) The continuing conundrum of the LEA proteins. *Naturwissenschaften*, **94**, 791–812.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl Acad. Sci. USA*, **97**, 11632–11637.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**. RESEARCH0034.

Wang, F. and Perry, S.E. (2013) Identification of direct targets of FUSCA3, a key regulator of Arabidopsis seed development. *Plant Physiol.* **161**, 1251–1264.

Wehmeyer, N. and Vierling, E. (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. *Plant Physiol.*, **122**, 1099–1108.

Whitmarsh, C.J. and Ortiz-Lopez, A. (2000) The de-greening of canola. *USDA Agr. Res. Mag.* **48**, 1.

Yamauchi, Y., Hasegawa, A., Taninaka, A., Mizutani, M. and Sugimoto, Y. (2011) NADPH-dependent reductases involved in the detoxification of reactive carbonyls in plants. *J. Biol. Chem.* **286**, 6999–7009.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *Plant J.* **61**, 672–685.

