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Alternative polyadenylation controls seed dormancy

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Research Area:
Genes, Development, and Evolution
Systems and Synthetic Biology

Seed dormancy in *Arabidopsis thaliana* is controlled by alternative polyadenylation of *DOG1*

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One-sentence summary:

DOG1 is alternatively polyadenylated and proximally polyadenylated *DOG1* is required for seed dormancy.

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Abstract

DOG1 is a key regulator of seed dormancy in Arabidopsis and other plants. Interestingly, the C-terminus of DOG1 is either absent or not conserved in many plant species. Here, we show that in Arabidopsis, *DOG1* transcript is subject to alternative polyadenylation. In line with this, mutants in RNA 3' processing complex display weakened seed dormancy in parallel with defects in *DOG1* proximal polyadenylation site selection, suggesting that the short *DOG1* transcript, is functional. This is corroborated by the finding that the proximally polyadenylated short *DOG1* mRNA is translated *in vivo* and complements the *dog1* mutation. In summary, our findings indicate that the short DOG1 protein isoform produced from the proximally polyadenylated *DOG1* mRNA is a key player in the establishment of seed dormancy in Arabidopsis and characterize a set of mutants in RNA 3' processing complex required for production of proximally polyadenylated functional *DOG1* transcript.

INTRODUCTION

The ability to postpone germination, to align it with permissive environmental conditions, greatly enhances the chances of plants survival (Koornneef et al., 2002; Penfield and King, 2009). Therefore, seed dormancy is extensively controlled by many factors including: humidity, temperature and light, and is also subject to parental memory (Chiang et al., 2011; Finch-Savage and Leubner-Metzger, 2006; Graeber et al., 2012; Nonogaki, 2014). *DOG1* (Delay of Germination 1) has been identified as a major QTL (Quantitative Trait Locus) for seed dormancy variability among natural Arabidopsis accessions and *dog1* T-DNA insertional mutants exhibit reduced seed dormancy (Bentsink et al., 2006). The expression of *DOG1* increases during seed maturation and the mRNA disappears quickly after imbibition, although the DOG1 protein is more stable (Nakabayashi et al., 2012). A recent report indicated that DOG1 enforces seed dormancy by strengthening the seed coat, which occurs via modulation of the expression of gibberellin metabolism genes (Graeber et al., 2014). The expression of *DOG1* is extensively regulated, being strongly induced by ABA (Abscisic acid) and low temperature during seed maturation (Chiang et al., 2011; Kendall et al., 2011). Other factors required for *DOG1* expression include histone-modifying enzymes such as the histone H2B ubiquitin transferase HUB (Liu et al., 2007) and H3 lysine 9 methyl transferase KYP (Zheng et al., 2012). *DOG1* expression is also highly dependent on transcription elongation factor TFIIS (Grasser et al., 2009).

The *DOG1* gene is comprised of 3 exons, and the exon 2-exon 3 junction is subject to alternative splicing, generating 4 different forms of mRNA (Bentsink et al., 2006). The function of these alternatively spliced transcript isoforms is unknown, and their relative ratio remains unchanged during seed development (Bentsink et al., 2006). Our recent analysis of the regulation of alternative splicing of *DOG1* in a mutant defective in PolII elongation suggested that the rate of transcript elongation regulates alternative splice site selection in accordance with the kinetic coupling model and implies that DOG1 splicing is co-transcriptional (Dolata et al., 2015).

To our knowledge complementation of the *dog1* mutation in Arabidopsis has not been achieved using a *DOG1* cDNA, while the seed dormancy phenotype of this mutant was complemented using a genomic *DOG1* clone from *Lepidium sativum* (*L. sativum*) (Graeber et al., 2014). Notably, the *L. sativum DOG1* gene lacks the exon 3,

so can only encode a short two-exonic mRNA, with no alternatively spliced exon 2-exon 3 isoforms.

Many alternatively spliced genes are also subject to alternative polyadenylation (APA) (Di Giammartino et al., 2011). Alternative polyadenylation leads to the generation of transcripts with different 3' ends, through a series of steps catalyzed by components of RNA 3’ processing complexes, like: the Cleavage and Polyadenylation Specificity Factor (CPSF) and Cleavage Stimulation Factor (CstF) complexes, and poly(A) polymerases (Gruber et al., 2014; Proudfoot, 2011; Mandel et al., 2008). APA is common and widespread in animals, plants and other eukaryotic organisms (Tian et al., 2005; Pickrell et al., 2010; Sun et al., 2012; Wu et al., 2015; Shi, 2012). In animals APA is involved in a range of developmental processes including cell differentiation and has been implicated in cancer (Danckwardt et al., 2008; Mayr and Bartel, 2009; Lianoglou et al., 2013; Lin et al., 2012). Similarly, in plants APA has been shown to control key developmental processes.

In one case, the nuclear RNA-binding protein FCA interacts with FY, a component of CPSF complex, to promote the usage of a proximal polyadenylation site in its own gene, leading to the production of a non-functional RNA isoform (Simpson et al., 2003). In addition, FCA functions with CstF complex to promote proximal polyadenylation of the non-coding antisense transcript of *FLC* (Flowering Locus C), leading to suppression of *FLC* expression (Liu et al., 2010). Alternative polyadenylation has also been implicated in the control of pathogen resistance in Arabidopsis through the selection of proximal polyadenylation sites in the *RPP7* (Recognition Of Peronospora Parasitica 7) gene (Tsuchiya and Eulgem, 2013).

Given the key function of DOG1 in Arabidopsis seed survival and the potential role of DOG1 homologs in controlling seed dormancy in other species, it is important to understand both the mechanisms of *DOG1* locus regulation and the function of the encoded protein. Here we describe the process of *DOG1* alternative polyadenylation that produce two alternatively polyadenylated isoforms of the *DOG1* transcript. We characterize mutants in proteins that control this mechanism, and show that the proximally polyadenylated mRNA isoform is translated *in vivo*, and is functional as it complements the *dog1* mutant. Demonstrating that alternative polyadenylation of *DOG1* gene in Arabidopsis plays a fundamental role in regulation of seed dormancy.

RESULTS

Mutants of RNA 3' processing factors display weak seed dormancy phenotype

The majority of Arabidopsis genes have multiple polyadenylation sites (Wu et al., 2011), but the biological consequences of the alternative polyadenylation are in most cases unknown. FY is the Arabidopsis homologue of the yeast RNA 3' processing factor *Pfs2p* (Simpson et al., 2003). A previous study demonstrated that the *fy-1* mutant in Landsberg erecta (Ler) background has weak seed dormancy (Jiang et al., 2012). To check if the function of FY in seed dormancy control is independent of genetic background, we assayed seed dormancy of *fy-2* mutant in Col-0 background. Compared to the Col-0 wild type, *fy-2* seeds showed very weak dormancy (Figure 1A) demonstrating that FY plays a significant role in controlling this process in Arabidopsis. Since FY functions with other RNA 3' processing factors in flowering time control (Liu et al., 2010; Manzano et al., 2009), we analyzed seed dormancy of the mutants of RNA 3' processing factors PCFS4 (PCF11P-Similar Protein 4) and ESP1 (Enhanced Gene Silence 1). Like *fy* mutants, the *pcsf4-1* and *esp1-2* mutants showed weakened seed dormancy (Figure 1B), suggesting that the observed seed dormancy defect is caused by misregulation of RNA 3' processing of a gene or genes involved in seed dormancy control.

Next, we examined whether the weak seed dormancy phenotype of *fy*-2 may be due to misregulation of *DOG1* transcription. RT-qPCR (reverse transcription and quantitative PCR) analysis revealed no significant change of *DOG1* mRNA level in *fy-2* mutant (Figure 1C). However, western blot analysis using a DOG1-specific antibody revealed a consistent reduction in the DOG1 protein level in *fy*-2 seeds when compared with Col-0 wild type (*Wt*) (Figure 1D and Supplemental Figure S1). Thus, we concluded that FY is required for proper DOG1 protein expression and hypothesized that the *fy*-2 mutant may be defective in *DOG1* RNA processing, which leads to the suppression of translation. Therefore, we searched for potential *DOG1* polyadenylation defects in *fy-2*. Surprisingly, 3' RACE experiments initiated from exon 3 of *DOG1* showed no obvious differences in either polyadenylation site selection or the length of the poly(A) tail, between Col-0 wild type and *fy*-2 seeds (Figure 1E).

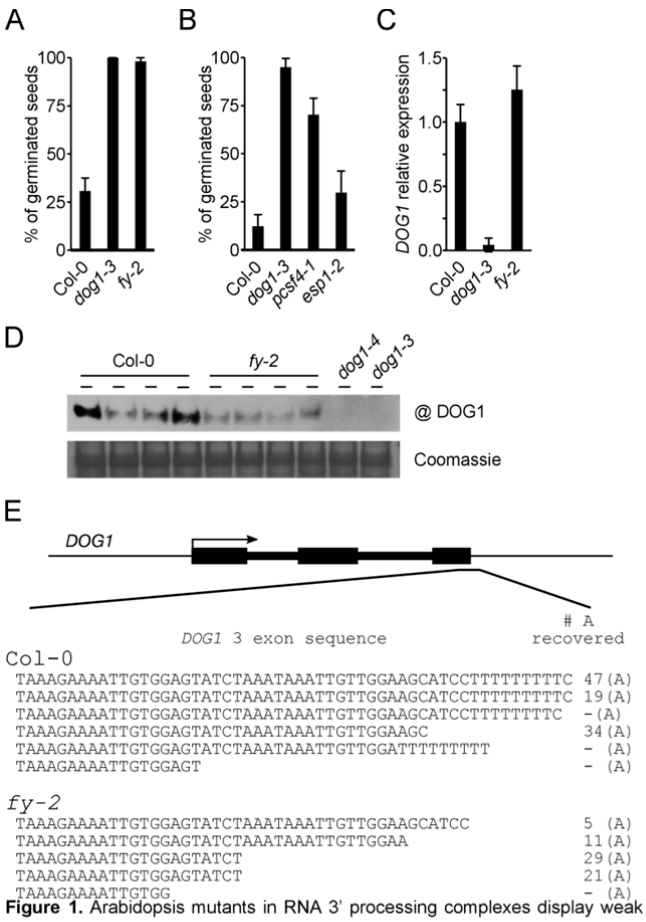

Figure 1. Arabidopsis mutants in RNA 3' processing complexes display weak seed dormancy, unchanged *DOG1* expression and a low DOG1 protein level.

(A) Germination efficiency (%) of freshly harvested seeds of *fy-2* mutant. The graph shows the mean values from at least four biological replicates, with error bars representing SD. Each experiment was repeated at least three times. *dog1-3* mutant shown as positive control.

(B) Germination efficiency (%) of freshly harvested seeds of RNA 3' processing mutants. The graph shows the mean values from at least four biological replicates, with error bars representing SD. Each experiment was repeated at least three times.

(C) Relative expression level of *DOG1* in freshly harvested seeds from Col-0, *dog1-3* and *fy*-2 plants determined by RT-qPCR. Values were normalized to the level of *UBC* mRNA and are the means of at least three biological replicates, with error bars representing SD.

(D) The *fy-2* mutant exhibits a decreased level of DOG1 protein in seeds. The DOG1 protein level was determined by western blotting in Col-0, *fy-2*, *dog1-3* and *dog1-4* mutant seed extracts using a polyclonal DOG1 antibody. The Coomassie blue-stained gel is shown as a loading control. Four biological replicates were analyzed for Col-0 and *fy-2* plants.

(E) 3' RACE initiated within *DOG1* exon3 reveals no clear defects in transcription termination. RACE was performed using RNA isolated from freshly harvested seeds of Col-0 and *fy-2* lines. The alignment shows the 3' end sequence of individual clones with the number of adenines (A) attached for each clone.

Alternative polyadenylation of *DOG1* gene results in production of two mRNA isoforms

As we were unable to detect any defect in the use of the canonical polyadenylation site of *DOG1* in the *fy* mutant, we searched for additional

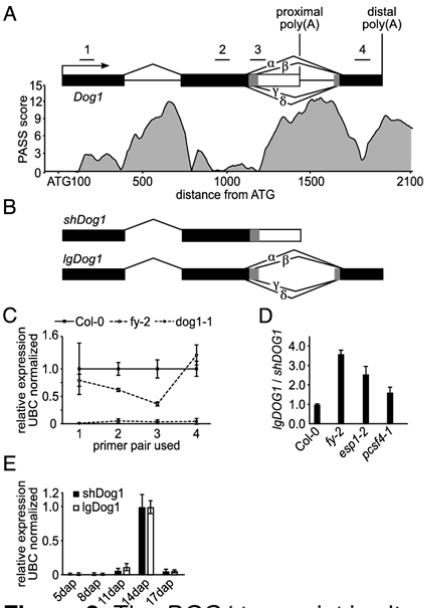

Figure 2. The *DOG1* transcript is alternatively polyadenylated.

(A) Polyadenylation site prediction along the *DOG1* gene performed with PASPA software. Diagram of *DOG1* gene structure with alternatively spliced isoforms of intron 2 labeled as alpha, beta, gamma and delta, plus the position of the alternative polyadenylation site. Lines above represent the fragments amplified in RT-qPCR analysis using the numbered primer sets. Below shown in scale with the *DOG1* gene structure is the diagram of the quality score representing the likelihood of a polyadenylation site predicted by PASPA software.

(B) Schematic presentation of two alternatively polyadenylated *DOG1* mRNA isoforms.

(C) RT-qPCR along the *DOG1* gene to examine the expression of the different mRNA isoforms in Col-0, *fy-2* and *dog1-3* seeds. Values were normalized to the level of *UBC* mRNA. Expression in mutants is normalized to the Col-0 value. Data represent the means of three biological replicates; error bars show SD.

(D) Long *DOG1*/short *DOG1* ratio in Col-0 and mutants of RNA 3' processing factors. RT-qPCR results were normalized to the level of *UBC* mRNA, and the expression in mutants is then normalized to the Col-0 value. Data represent the means of three biological replicates with error bars showing SD.

(E) Alternative *DOG1* polyadenylation site selection during seed development. RT-qPCR specific for *shDOG1* and *lgDOG1* was used to measure relative levels of each mRNA isoform. Manually pollinated flowers were collected at different days after pollination (dap). Values were normalized to the level of *UBC* mRNA. Data represent the means of three biological replicates with error bars showing SD.

polyadenylation sites within this gene. The most common polyadenylation signal motif in plants is AAUAAA and UUGUUU positioned 19 and 7 nt respectively upstream of the cleavage site (Sherstnev et al., 2012). The identification of likely polyadenylation sites with PASPA (Ji et al., 2015), a software designed for mRNA

polyadenylation site prediction in plants, revealed three potential polyadenylation clusters in the *DOG1* gene. One cluster corresponds to the predicted full length polyadenylation site of *DOG1*, and the two others to additional internal polyadenylation sites: one in intron 1 and another in intron 2 (Figure 2A). To validate these predictions, we reanalyzed published Direct RNA Sequencing based mapping data to detect Arabidopsis polyadenylation sites in the *DOG1* locus (Supplemental Figure S2) (Sherstnev et al., 2012). We identified two distinct polyadenylation clusters: a distal one used to produce the predicted full-length mRNA, which is named hereafter as the long *DOG1* (*lgDOG1*) form, and a proximal one matching one of the predicted internal polyadenylation sites located in intron 2 that would produce a truncated mRNA, which we name short *DOG1* (*shDOG1*). The second predicted internal polyadenylation site lies within intron 1, but analysis of RNA sequencing data revealed no indication of its usage (Supplemental Figure S2).

In summary, the *lgDOG1* transcript is comprised of three exons and corresponds to the previously described Arabidopsis *DOG1* mRNA (Bentsink et al., 2006), whereas the newly identified *shDOG1* transcript comprises only exons 1 and 2, and therefore lacks the alternative splicing isoforms described (Figure 2B).

Next, to quantify the different *DOG1* mRNA isoforms in Col-0 wild type and *fy-2* mutant seeds we designed sets of primers within the *DOG1* locus for use in RT-qPCR analysis. Amplification with primer set 4 showed no difference in the level of *lgDOG1* mRNA between *fy-2* and wild type seeds (Figure 2A and Figure 2C). In contrast, use of primer set 3 demonstrated a clear reduction of the *DOG1* transcript in *fy-2* seeds. The primers of set 3 span the exon 2-intron 2 border that is specific for the short alternatively polyadenylated *shDOG1* mRNA isoform. Therefore, this result suggested a reduction in usage of the proximal polyadenylation site in the *fy*-2 mutant. To confirm this, we designed primers to amplify the full-length short and long *DOG1* transcript isoforms and obtained similar results (Supplemental Figure S3).

Different mRNA isoforms generated through APA often differ in their stability (Krol et al., 2015). However, a cordycepin-dependent RNA stability assay (Golisz et al., 2013) showed that both the short and long *DOG1* mRNA isoforms have a similar half-life of about 1 h (Supplemental Figure S4). This shows that usage of *DOG1* proximal polyadenylation site leads to the production of a stable transcript.

A change in the long/short *Dog1* transcript ratio, similar to that observed in *fy*-2, was also detected in mutants of other factors required for RNA 3’ processing,

which showed weak seed dormancy (Figure 2D and Figure 1B). This finding led us to speculate that the reduction in the *shDOG1* mRNA level in these mutants, may be the underlying cause of their reduced seed dormancy.

Having established that the *DOG1* gene is subjected to alternative polyadenylation, we questioned whether this process is developmentally regulated. *DOG1* expression is tightly controlled during seed development and has previously been shown to peak between 9-16 days after pollination and then slowly decay (Nakabayashi et al., 2012; Zhao et al., 2015). Alternative splicing of the *lgDOG1* mRNA isoform has been reported to be unaffected during seed development (Bentsink et al., 2006). Our analysis of short and long *DOG1* mRNA levels showed that both forms are induced during seed development and show a similar expression profile (Figure 2E).

Short *DOG1* mRNA is translated *in vivo* and produce a conserved protein

The previously recognized long *DOG1* mRNA transcript (*lgDOG1*) encodes a protein of ~32 kD. The proximally polyadenylated *shDOG1* transcript described in this study codes for a slightly smaller ~30 kD protein. These short and long DOG1 proteins only differ by several amino acids at their C-terminal ends, encoded by exon 3 in *lgDOG1* and a sequence from intron 2 in *shDOG1* (Figure 3A). Interestingly, a multiple sequence alignment with DOG1 proteins from other species showed that the sequence unique to shDOG1 is conserved at a similar level compared to that encoded by exon 2, while the amino acids unique to lgDOG1 (exon 3) are either absent due to stop codons or are weakly conserved (Figure 3A). Evolutionary conservation is often considered an indication of functionality, suggesting that the shDOG1 protein is functional.

Next, we determined which of the *DOG1* mRNA isoforms is translated. DOG1 antibodies used previously and that described here do not distinguish the long and short DOG1 proteins because they were raised to peptides shared by the two isoforms (Nakabayashi et al., 2012). In addition, the small difference in protein mass does not allow distinction between the shDOG1 and lgDOG1 proteins on a western blot: only one DOG1 band is observed in samples from freshly harvested seeds (Figure 1D). Therefore, we created a *GFP*::*DOG1* genomic fusion driven by the *DOG1* promoter. Samples from transgenic plants expressing this fusion protein were used in a GFP pull-down assay (Figure 3B) with subsequent analysis by mass

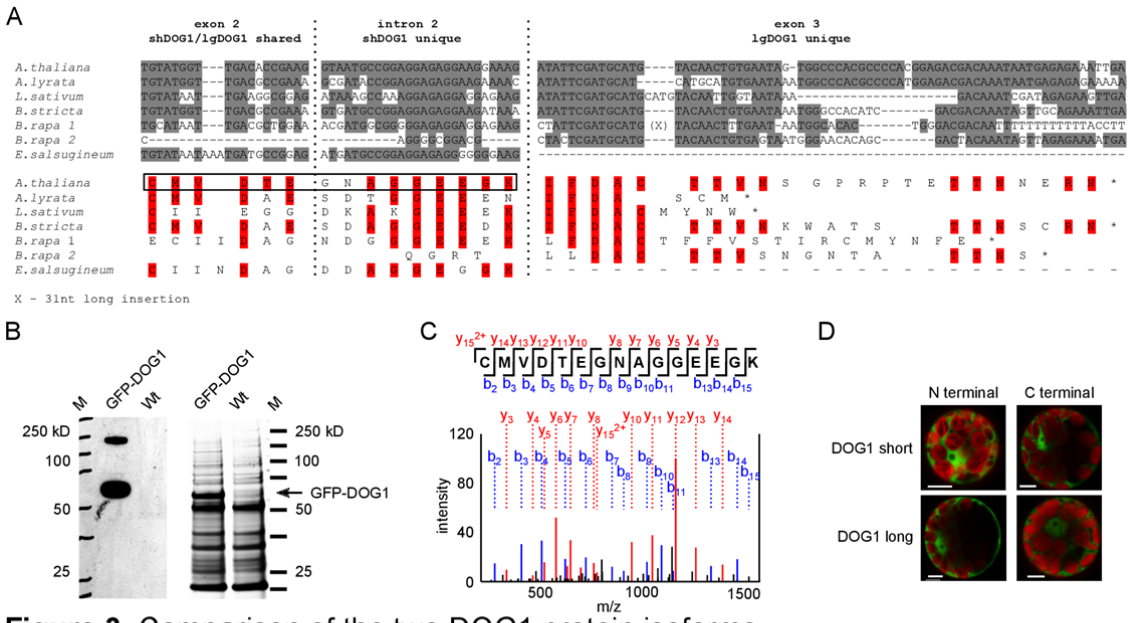

Figure 3. Comparison of the two DOG1 protein isoforms

(A) Alignment of *DOG1* genomic sequences from selected plant species used to construct the protein sequence alignment beneath. Nucleotides and amino acids shared with *A. thaliana* are highlighted by grey and red shading, respectively. Black box highlights peptide unique to shDOG1 identified below.

(B) Purification of DOG1 from green siliques extract of plants expressing a GFP-DOG1 fusion under the control of the native promoter and Col-0 (Wt) plants, using GFP Nano-trap beads. Isolated proteins were analysed with western blot using GFP specific antibodies (left panel) and on a SDS-PAGE gel followed by silver staining (right panel). GFP-DOG1 band is marked with an arrow.

(C) Purified proteins were subjected to mass spectrometry analysis. Short DOG1 specific peptide fragmentation with MS spectra of fragments derived, shown as intensity (Y-axis) sorted by mass-to-charge ratio (m/z) shown on X-axis.

(D) Short and long DOG1 show nuclear localization. Arabidopsis protoplasts were transformed with constructs encoding N- and C- terminal fusions of GFP with long and short DOG1, then analyzed using a confocal microscope. The green colour represents GFP and red the chlorophyll signal.

spectrometry. Notably, no long DOG1-specific peptides were identified, but we could clearly detect peptides unique to the shDOG1 protein (Figure 3C and Supplementary Figure S5). Although this does not exclude the possibility that the long DOG1 form is expressed but not detected, this result clearly showed that use of the proximal

polyadenylation site leads to the production of a short *DOG1* mRNA that is translated *in vivo*.

A previous study examining Arabidopsis lines constitutively overexpressing DOG1 suggested nuclear localization of this protein (Nakabayashi et al., 2012). However, this experiment did not distinguish between the short and long DOG1 forms. Therefore, we compared the localization of these DOG1 proteins. Both N- and C-terminal GFP fusions of the shDOG1 and lgDOG1 protein showed predominantly nuclear localization when transiently expressed in Arabidopsis protoplasts (Figure 3D).

We conclude that the short alternatively polyadenylated *DOG1* mRNA isoform is translated *in vivo*, thus producing a short nuclear localized DOG1 protein. The fact that the shDOG1 is conserved whereas the lgDOG1 is less together with our inability to find long DOG1 specific peptides suggested that the shDOG1 maybe the functional DOG1 protein in respect to seed dormancy.

Short DOG1 is sufficient to inhibit germination

To directly test the ability of the short and long DOG1 proteins to control seed dormancy in Arabidopsis we created *dog1-3* mutant plants with constructs carrying the *shDOG1* and *lgDOG1* sequences driven by the *DOG1* promoter (Figure 4A). Only the *shDOG1* construct was able to partially complement the weak seed dormancy phenotype of *dog1-3* (Figure 4A), even though both showed a similar *DOG1* expression as measured by qRT-PCR (Supplementary Figure S6). This indicated that shDOG1 protein is sufficient for seed dormancy establishment. To further support this conclusion we selected a *dog1-5* T-DNA insertion mutant allele. In this mutant a T-DNA is inserted in exon 3 of the *DOG1* gene (Figure 4B). RT-qPCR analysis with primers located at the exon 2-exon 3 junction, upstream of the T-DNA insertion, confirmed that *lgDOG1* is no longer expressed in this mutant (Figure 4C). In contrast, levels of the *shDOG1* transcript appear slightly increased in *dog1-5.* Accordingly, the *dog1-5* mutant exhibited an increase in the DOG1 protein level, and increased rather than reduced seed dormancy (Figure 4D and Figure 4E). These findings confirmed that the short version of the *DOG1* transcript generated through APA is translated and functional in controlling seed dormancy.

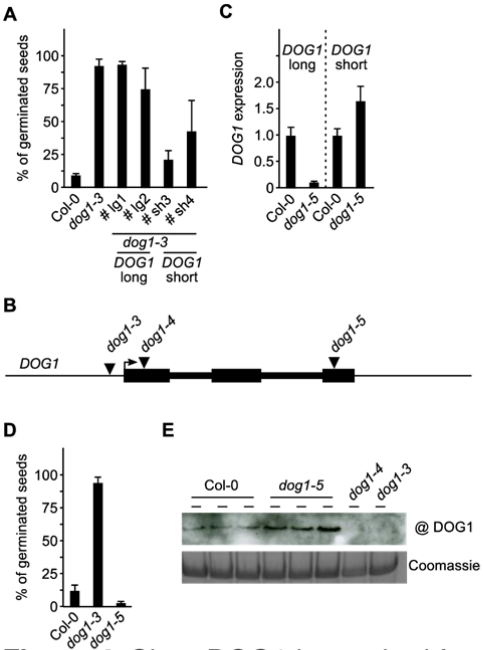

Figure 4. Short DOG1 is required for seed dormancy.

(A) Germination rate (%) of freshly harvested seeds of the *dog1-3* mutant and *dog1-3* complemented with long (# lg1, # lg2) and short (# sh3, # sh4) versions of the *DOG1* gene controlled by its native promoter. Data represent the means of at least 4 biological replicates with error bars showing SD.

(B) Schematic description of *DOG1* mutants: *dog1-3* (SALK_000867), *dog1-4* (SM_3_20886) and *dog1-5* (SALK_022748).

(C) Relative expression level of long and short *DOG1* mRNAs in freshly harvested seeds from Col-0 and *dog1-5* plants determined by RT-qPCR. Values were normalized to the level of *UBC* mRNA. Expression in the mutant is normalized to the Col-0 value. Data represent the means of three biological replicates; error bars show SD.

(D) Germination rate (%) of freshly harvested seeds of *dog1* mutants. The graph shows the means from three biological replicates, with error bars showing SD. The experiment was repeated at least three times.

(E) Western blot analysis of the DOG1 protein level in freshly harvested seeds from Col-0, *dog1-5, dog1-4* and *dog1-3* plants. The Coomassie blue-stained gel is shown as a loading control. Three biological replicates were examined for Col-0 and *dog1-5* plants.

DISCUSSION

Alternative polyadenylation is widespread in eukaryotes. Whole genome polyadenylation site mapping has revealed that the vast majority of Arabidopsis genes harbour alternative polyadenylation sites, but the biological significance of this phenomenon is mostly unknown. In this study, we have shown that the Arabidopsis *DOG1* gene is subjected to alternative polyadenylation. The proximally polyadenylated short *DOG1* transcript is translated and functional, in contrast to the long *DOG1*. Our data clearly demonstrate that expression of the short *DOG1* isoform is sufficient to promote seed dormancy, thus providing an example where alternative polyadenylation is important in seed development.

DOG1 mRNA is alternatively polyadenylated

DOG1 is a major QTL for seed dormancy in Arabidopsis and an important regulator of this process in agriculturally important plants. Here, we show that the Arabidopsis *DOG1* gene produces two alternatively polyadenylated RNAs. The production of the short isoform is sensitive to mutations in RNA 3' processing complex components. RNA 3' processing complex mutants with defects in selection of the proximal polyadenylation site on *DOG1* show weak seed dormancy, suggesting that the proximal polyadenylation is required for *DOG1* function. This is corroborated by the finding that the RNA 3' processing mutant *fy-2* displays low DOG1 protein levels, but no change, or increase, in the level of the full-length *DOG1* transcript. Taken together, these data indicate that the short alternatively polyadenylated *DOG1* isoform is functional in seed dormancy regulation. The most well characterized, developmental processes that involve a critical change in the polyadenylation site, is the global switch to the usage of proximal polyadenylation sites observed during mammalian brain development or in tumors, which has been proposed to cause a release from miRNA mediated control due to the loss of miRNA binding sites (Elkon et al., 2013; Di Giammartino et al., 2011). This is unlikely to be the underlying reason for the switch in polyadenylation site of *DOG1* since there is no dramatic difference in RNA stability between the long and short *DOG1* isoforms.

Short DOG1 protein is functional in seed dormancy establishment

A reanalysis of published data together with our results show that the *DOG1* locus produces two alternatively polyadenylated forms of *DOG1* mRNA. Interestingly, the *DOG1* genes in *A. lyrata* and *L. sativum*, two species quite closely related to Arabidopsis, harbour mutations, including stop codons in exon 3, that encodes the specific C-terminal region of the long DOG1 isoform. This shows that these species express only the short DOG1 protein isoform encoded by exons 1 and 2 plus part of intron 2.

Our complementation analysis demonstrated that while the long DOG1 is unable to complement the *dog1-3* mutation, the short version of DOG1 is functional. In addition, we identified a novel mutant *dog1-5,* carrying a T-DNA insertion in exon 3, a region that is only present in *lgDOG1*. This mutant lacks the long version of the *DOG1* transcript but it expresses the proximally polyadenylated *shDOG1* at higher level than wild type and exhibits stronger seed dormancy. This confirms that the shDOG1 isoform produced by alternative polyadenylation in intron 2 is sufficient for seed dormancy.

The fact that the *fy*-2 mutant shows a lower level of the DOG1 protein without any reduction in the *lgDOG1* mRNA strongly suggests that this transcript does not produce a protein in this mutant. Examination of currently available proteomic datasets revealed DOG1 peptides. However all of them correspond to *DOG1* exons 1 and 2, and none are specific to either the short or long DOG1 protein isoforms (Baerenfaller et al., 2008; Castellana et al., 2008). Nevertheless, using targeted mass spectrometry analysis of immunoprecipitated DOG1-GFP, shDOG1-specific peptides were clearly detectable, but we did not identify peptides specific for lgDOG1.

The short DOG1 protein can be derived either through the use of a proximal polyadenylation site or by splicing of the long *DOG1* transcript at an alternative splice site (*DOG1*-beta mRNA isoform) (Bentsink et al., 2006). Analysis of splice sites in the *DOG1* gene reveals weak conservation of the exon 3 acceptor splice site used by the *DOG1*-beta mRNA isoform. This, together with the strong DOG1 protein signal in mutant *dog1-5* that lacks the *lgDOG1* mRNA isoforms, suggests that the shDOG1 protein is predominantly produced from the proximally polyadenylated short *DOG1* transcript isoform.

The function of the long *DOG1* transcript and lgDOG1 protein is currently not clear. Lack of conservation of the lgDOG1 protein specific sequence encoded by exon 3, together with the inability of *lgDOG1* to complement *dog1* mutant suggest

that long DOG1 protein may be not functional. However, extensive examples of alternative polyadenylation sites competition, including proximal and distal FCA polyadenylation sites (Simpson et al., 2003), imply that the *DOG1* distal polyadenylation site might acts as a decoy for transcripts that fail to terminate earlier. If so, then it could be expected that the *lgDOG1* mRNA is rapidly degraded. Due to technical limitations we were unable to perform RNA stability assays on intact seeds. However, mRNA half-life measurements in seedlings showed no clear difference between the stability of the long and short *DOG1* transcripts.

It is interesting to note, that the *dog1-5* mutant shows upregulation of both the proximally polyadenylated *shDOG1* mRNA isoform and the DOG1 protein, suggesting that the *lgDOG1* mRNA may act as a negative regulator of *shDOG1* mRNA expression.

In summary, our findings provide several lines of evidence demonstrating that *DOG1* nascent transcript is subjected to alternative polyadenylation and that the short form of this transcript is functional in seed dormancy establishment. Given that DOG1 is subject to elaborate transcriptional elongation control it would be interesting to test whether polyadenylation site selection is controlled by the rate of PolII elongation on *DOG1*, as is the case for splice site selection (Dolata et al., 2015).

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METHODS

Plant materials and growth conditions

Arabidopsis thaliana plants were grown on soil in a greenhouse with a 16-h light/8-h-dark photoperiod at 22°C/18°C. For expression analysis during seed development, flowers were emasculated and pollinated the following day. Plant material was collected at the indicated times representing days since manual pollination. The termination complex mutants used in this study have been described previously: *fy-2* (Simpson et al., 2003), *esp1-2* (SALK_078793), *pcsf4-1* (SALK_102934c) (Xing et al., 2008). Col-0 was used as *Wt* control. The *DOG1* T-DNA insertion mutants used were: *dog1-3* (SALK_000867) (Bentsink et al., 2006), *dog1-4* (SM_3_20886) and *dog1-5* (SALK_022748).

Germination assay

About 100-150 freshly harvested Arabidopsis seeds were sown on blue germination paper (Anchor) supported by 2 layers of thick fabric saturated with water. Plates were sealed and transferred to a growth chamber with 16h/8h light/dark at 22°C/18°C. Plates were photographed daily for 7 days and germinating seeds counted. At least three biological replicates were performed for each experiment. Data show a time point when the *dog1-3* mutant seed had fully germinated. Seed dormancy strength is strongly dependent on external conditions during seed maturation (Kendall et al., 2011) we therefore always included a set of controls: *dog1-3* and WT in all experiments. Small external condition fluctuations between separate experiments may therefore explain the difference in seed germination strength between Col-0 plants in Figure 1A and 1B.

RNA extraction, cDNA synthesis and PCR analysis

Total RNA was isolated using a phenol-chloroform extraction procedure (Shirzadegan et al., 1991). The RNA was then treated using a TURBO DNA-free™ Kit (Life Technologies), according to the manufacturer's protocol. The efficiency of DNA removal was monitored by *PP2A* PCR. Before reverse transcription, the RNA was examined by electrophoresis on a 1.2% agarose gel to determine its quality and quantified using a Nanodrop 2000 spectrophotometer. For cDNA synthesis, 2.5 µg of RNA were used with oligo(dT) primers and a RevertAid™ First Strand cDNA

Synthesis Kit (Fermentas). Diluted cDNA (10-fold) was used in qPCR (LightCycler ® 480 Roche), with SYBR Green mix (Roche). A *UBC* gene was used for normalization (Czechowski et al., 2005).

Protein extraction

Freshly harvested Arabidopsis seeds (30 mg) were frozen in liquid nitrogen and pulverized in 100 µl of acetone. The suspension was centrifuged for 1 min at 1000 g. The pelleted material was rinsed with 96% EtOH and centrifuged again as above. The pellet was then resuspended in 50% Percoll and centrifuged for 10 min at 1000 g. Pelleted material was again washed in 96% EtOH and centrifuged for 1 min at 1000g. The final pellet was resuspended in 8 M urea for analysis by SDS-PAGE.

Western blotting

Proteins were extracted from freshly harvested seeds as described above. The protein concentration was determined using the Bradford method (Bradford, 1976) and samples were analyzed by standard SDS-PAGE. Proteins were then electrophoretically transferred to Hybond P membrane (Amersham) using a Trans-Blot Cell (Bio-Rad). A synthetic peptide (MGSSSKNIEQAQDSY) corresponding to amino acids 1 to 15 of DOG1 was synthesized and used to produce an affinity purified DOG1-specific rabbit polyclonal antibody (Eurogentec). DOG1-specific (Eurogentec) and Goat Anti-Rabbit IgG H&L (HRP) (Abcam b7090) antibodies were also used for western blotting. Western blot was developed using a 1:1 mixture of SuperSignal West Pico and Femto Chemiluminescent Substrates (Pierce).

GFP-DOG1 Immunoprecipitation (IP) and mass spectrometry analysis

pDOG1::GFP::DOG1g vector was constructed using primers shown in Supplementary Table 1 and a pCambia5408 modified to carry a Basta resistance under NOS promoter resulting in native *DOG1* gene with GFP inserted at ATG. Plant extracts from mixture of green and mature siliques of transgenic plants were used for IP with GFP Nano-trap beads (ChromoTek). MS followed by analysis using Mascot software was performed as described previously (Dolata et al., 2015).

Protoplast transformation

The following plasmid constructs were prepared in the vectors pSAT6-eGFP-C1 and pSAT6A-eGFP-N1 using the short and long *DOG1* cDNAs: 35S::sDOG1-YFP, 35S::YFP-sDOG1, 35S::lgDOG1-YFP and 35S::YFP-DOG1. To maximize the efficiency of protoplast isolation protoplasts were isolated from the leaves of 20-d-old late flowering Col-0 with backcrossed active FRI (Frigida) from SF2 ecotype (Michaels and Amasino, 1999), and transformed with 30 µg of purified construct DNAs according to a previously described method (Wu et al., 2009). Transformed protoplasts were incubated overnight under continuous light at 22°C prior to analysis by confocal microscopy.

Confocal microscopy imaging

Fluorescence images of transformed Arabidopsis protoplasts were visualized using a Nikon EZ-C1 laser scanning microscope mounted on an inverted Nikon TE 2000E epifluorescence microscope. Excitation was at 514 nm, and the emission signal was collected between 525 and 590 nm for YFP fluorescence, and between 622 and 700 nm for chlorophyll autofluorescence. Untransformed protoplasts were examined as a negative control.

3' RACE

RACE was performed as described previously (Swiezewski et al., 2009). Total RNA from seedlings was ligated to 3' RACE oligo adapter (Gene Racer kit, Invitrogen) using T4 RNA Ligase 1 (New England Biolabs). After phenol/chloroform extraction RNA was precipitated and subjected to reverse transcription using adapter-specific primer and Superscript III (Invitrogen). 3' ends of DOG1 mRNA were amplified using adapter specific primer and DOG1 specific RACE primer. PCR products were purified from agarose gel and cloned to pJet1.2 vector and sequenced using plasmid specific primers in both directions.

RNA stability assay

Cordycepin dependent RNA stability assay was performed as previously described (Golisz et al., 2013). In brief, Col-0 plants were grown on ½ MS medium containing 1% sucrose in a 16-h light/8-h-dark cycle at 22°C. About 100 two-week-old seedlings were transferred to 40 ml of buffer (1 mM PIPES pH 6.25, 1 mM Trisodium citrate, 1

mM KCl, 15 mM sucrose) and incubated for 30 min with shaking. Cordycepin was then added to a final concentration of 150 µg/ml and vacuum infiltration performed for 30 s. At each time point thereafter, seedlings representing ~0.5 g were collected and frozen in liquid nitrogen. Samples were analyzed in triplicate. RNA extraction was performed as described above and RT-qPCR analysis with EIF4A and At3g45970 was used mRNAs with respectively high and low stability (Golisz et al., 2013)

dog1-3 **complementation**

For complementation with *lgDOG1*, *dog1-3* mutatant was transformed with a *DOG1* genomic fragment with the sequence of the second intron deleted. For complementation with *shDOG1*, the *dog1-3* mutant was crossed with Col-0 plants expressing a construct containing the genomic fragment spanning *DOG1* exon 1-intron 1 and exon 2. The *DOG1* genes in both constructs are driven by the endogenous *DOG1* promoter. Two independent transformants were analyzed for each construct.

Accession numbers

The sequences of genes examined in this study can be found in The Arabidopsis Information Resource (TAIR) data library under the following accession numbers: *DOG1* (At5g45830), *FY (*At5g13480*), ESP1* (At1g73840), *PCSF4* (At4g04885).

Supplemental Data

Supplemental Figure S1. Western blot and Coomassie-stained gel used for Figure 1D.

Supplemental Figure S2. *DOG1* polyadenylation site identified using Direct RNA sequencing data (Sherstnev et al., 2012).

Supplemental Figure S3. RT-PCR to examine the expression of full-length *shDOG1* and *lgDOG1* mRNA transcripts in Col-0 and *fy-2* seeds.

Supplemental Figure S4. RNA stability assay of *shDOG1* and *lgDOG1* transcripts.

Supplemental Figure S5. Characterization of short DOG1-specific peptides.

Supplemental Figure S6. *DOG1* expression in freshly harvested seeds of Col-0 (WT) and *dog1-3* or *dog1-3* mutants complemented with *lgDOG1* (# lg1, # lg2) or *shDOG1* (# sh3, # sh4) expressing constructs.

Supplemental Table 1. List of primers.

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AUTHOR CONTRIBUTIONS

S.S. and F.L. designed the project and planned the program of research. H.F., M.C. and A.S. performed the germination tests and expression level measurements. H.F. and M.C. performed the western blots. L.B. did the RACE experiment. S.S. and S.K. performed the bioinformatics analysis. A.C. and H.F. examined localization. G.Y. performed the DOG1-GFP IP. L.B. and A.S. performed the RNA stability assay. K.K and Z.P. prepared the short and long *DOG1* lines for complementation. S.S. and M.C. wrote the manuscript.

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