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Antisense transcription represses *Arabidopsis* seed dormancy QTL *DOG1* to regulate drought tolerance

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Abstract

Plants have developed multiple strategies to sense the external environment and to adapt growth accordingly. *Delay of germination 1* (*DOG1*) is a major quantitative trait locus (QTL) for seed dormancy strength in *Arabidopsis thaliana* that is reported to be expressed exclusively in seeds. *DOG1* is extensively regulated, with an antisense transcript (*asDOG1*) suppressing its expression in seeds. Here, we show that *asDOG1* shows high levels in mature plants where it suppresses *DOG1* expression under standard growth conditions. Suppression is released by shutting down antisense transcription, which is induced by the plant hormone abscisic acid (ABA) and drought. Loss of *asDOG1* results in constitutive high-level *DOG1* expression, conferring increased drought tolerance, while inactivation of *DOG1* causes enhanced drought sensitivity. The unexpected role of *DOG1* in environmental adaptation of mature plants is separate from its function in seed dormancy regulation. The requirement of *asDOG1* to respond to ABA and drought demonstrates that antisense transcription is important for sensing and responding to environmental changes in plants.

Keywords abscisic acid signalling; *DOG1*; drought stress; non-coding antisense RNA regulation

Subject Categories Plant Biology; RNA Biology; Signal Transduction

DOI 10.15252/embr.201744862 | Received 19 July 2017 | Revised 8 September 2017 | Accepted 15 September 2017 | Published online 13 October 2017

EMBO Reports (2017) 18: 2186–2196

Introduction

Delay of germination 1 (*DOG1*) was initially characterized as a QTL for seed dormancy variability between selected *Arabidopsis thaliana* accessions [1]. In agreement, *DOG1* has been reported to be exclusively expressed in seeds and in *Arabidopsis dog1* loss-of-function mutants displaying only seed dormancy-related phenotypes [1,2]. Subsequent studies have demonstrated that the seed dormancy function of *DOG1* is conserved in many different plant species [3–6]. Recent work has also shown that multiple independently evolved *DOG1* alleles are responsible for the adaptation of *Arabidopsis* to local conditions, explaining the surprisingly high proportion of naturally occurring variability in dormancy [7].

DOG1 expression is highly controlled in seeds, with regulators targeting *DOG1* alternative splice site selection [8], alternative polyA site selection [9] and *DOG1* expression [10–13]. In addition, we recently described the regulatory activity of a long, presumably non-coding antisense transcript that suppresses *DOG1* expression in "*cis*" and thereby dormancy strength in seeds [14].

Despite its predominant seed-specific expression, *DOG1* has been repeatedly identified in genomewide association studies (GWAS) as a candidate for controlling flowering time in *Arabidopsis* [15–17]. In agreement with this notion, RNAi-based silencing of *DOG1* in wheat and lettuce revealed not only defects in seed dormancy, but also in flowering phenotype [3,5]. However, flowering defects have not been observed in *Arabidopsis dog1* knockout plants [1,5].

All tested *DOG1* mRNA isoforms show primarily seed-specific expression [1,9,18], whereas the *DOG1* antisense transcript (*asDOG1*) is most strongly expressed in seedlings [14]. The *asDOG1* transcript originates from the 3′ end of *DOG1* close to the major polyA site [14,18], and it appears to be a member of a group of long, presumably non-protein-coding, RNAs (lncRNA). For recent reviews, see [19,20].

Antisense transcription from within terminators is a widespread and conserved phenomenon [21]. Yeast terminators that serve as promoters for antisense transcription share features that are typical for canonical sense promoters, including high H3K4me3 levels and the presence of TATA-like elements [22,23], but the functions of these transcripts are not always clear. Antisense transcription in yeast has been shown to permit the sensing of inorganic phosphate, lithium and many other stimuli [24–28].

Antisense transcription is well known in plants [29,30]. We recently demonstrated that some of these transcripts are initiated from within terminators which, as in yeast, show similarity to canonical promoters, including the presence of TATA boxes [31]. Importantly, many of the ncRNAs found in plants, including antisense transcripts, are extensively regulated by external and internal inputs. One example is *COOLAIR*, the antisense partner of *FLC*, which is upregulated by low temperature [32]. Thus, data from both

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yeast and *Arabidopsis* indicate that antisense transcripts can serve to regulate the expression of linked protein-coding genes in response to environmental conditions. For example, *FLC* antisense, redundantly with other players, was shown to be important for control of *FLC* regulation in response to cold [32,33].

Here, we show that *asDOG1* expression is strongly suppressed by both the plant hormone abscisic acid (ABA) and drought, resulting in the release of antisense-dependent silencing of *DOG1*. This discloses a role of *Arabidopsis* seed dormancy QTL *DOG1* in drought response as *dog1* mutants are drought-sensitive, and *asDOG1*-deficient plants (with constitutively high *DOG1* expression) are drought-resistant. Finally, we demonstrate that the ability of the antisense promoter to respond to ABA is absolutely required for the regulation of *DOG1* expression by this hormone. In summary, by dissecting the regulation of *asDOG1* by external stimuli, we have uncovered a novel and unexpected function of the major *Arabidopsis* seed dormancy QTL in drought response. Moreover, this study provides further evidence that antisense-mediated regulation of gene expression is important in plant responses to environmental cues.

Results

DOG1 antisense is highly expressed in *Arabidopsis* leaves

We have previously shown that *DOG1* antisense (*asDOG1*) negatively regulates *DOG1* sense expression in seeds. Notably, *asDOG1* is relatively weakly expressed in seeds and shows much higher expression in seedlings [14].

To thoroughly analyse *asDOG1* expression in different organs of *Arabidopsis,* we examined luciferase (LUC) activity in plants containing the IRES-LUC cassette driven by the *asDOG1* promoter—p_{AS}*DOG1::LUC* [14]. We found that the *asDOG1* promoter is highly active in the apical meristem, flowers and young leaves, but shows progressively diminishing activity in older leaves (Fig EV1). A complementary analysis using plants expressing a transgene containing the *LUC* reporter gene fused with the full-length *DOG1* locus—p_{DOG1}*LUC::DOG1*—revealed that *DOG1* mRNA is very weakly expressed in tissues other than seeds, with expression detected in the meristem, flowers and young leaves and diminishing activity in older leaves (Fig EV1). Analysis of plants expressing p_{DOG1}*shDOG1::LUC*, with deletion of the 3′ *DOG1* region (Δ antisense), revealed a slightly different expression pattern, with LUC activity detected mainly in older leaves, only a very weak signal in young leaves and no signal in flowers (Fig EV1B and D).

Thus, in agreement with our previous report, *asDOG1* shows strong expression in the meristem and leaves [14]. Furthermore, its expression is highly tissue-specific and similar to that of *DOG1* sense mRNA. The tissue specificity of antisense transcripts, or more broadly lncRNA, has been often reported in plants [29,30] and in other organisms [34–36]. The expression of many of these non-protein-coding transcripts is not only tissue-specific, it is also extensively regulated by the external environment [37,38]. This prompted us to examine the ability of the *asDOG1* promoter to respond to external stimuli. Importantly, *DOG1* antisense has been detected in *Arabidopsis* leaves in high-throughput direct RNA sequencing analysis [39,40], as shown previously [14].

DOG1 antisense is strongly downregulated by the hormone ABA

We challenged p_{AS}*DOG1::LUC* plants with the plant hormones ABA and gibberellin (GA), which both perform essential functions in seed dormancy and vegetative growth [41–44]. Treatment with ABA resulted in nearly complete silencing of the antisense promoter activity in leaves, compared to mock- or GA-treated plants (Figs 1A and B, and EV2A and B, Appendix Fig S1). ABA plays an important role in the response of plants to stress, including water deprivation where it acts as part of a signalling cascade leading to drought resistance [45–47]. Subsequently, we showed that the *DOG1* antisense promoter was also strongly downregulated in response to 5 days of water withdrawal (Figs 1C and D, and EV3A–C), a result validated by strand-specific RT–qPCR analysis in Col-0 (WT) plants (Fig 1E).

We next analysed how *asDOG1* expression responds to ABA in Col-0 (WT) plants by using RT–qPCR to monitor mRNA levels. In agreement with our initial observations (Fig 1A and B), we detected a strong (approx. 80%) reduction in *asDOG1* transcript abundance 10 h after ABA treatment. Simultaneously, we saw a ten-fold increase in the level of the short functional form of *DOG1* mRNA (Fig 1F).

Thus, *asDOG1* displays a tissue-specific expression pattern and is regulated by external signals. This response is selective, since the antisense expression was strongly downregulated by ABA but was not affected by GA (Fig 1B and Appendix Fig S1). The concomitant downregulation of antisense and upregulation of sense expression in response to ABA suggest that these processes are interconnected, but do not allow the causative factor to be singled out (Fig 1F).

Mutation of the *DOG1* gene causes enhanced sensitivity to drought

The strong upregulation of *DOG1* expression following ABA treatment suggests that this gene may perform some unrecognized novel functions during stress. This notion received support from the finding that *DOG1* knockout plants (*dog1-3* and *dog1-4*, described in [1,9,14] and shown in Fig EV1E), displayed a drought-sensitive phenotype in comparison with Col-0 (WT) plants. In the 15 plants of each line subjected to water deprivation, 14 of the WT recovered, while, respectively, none and one of the *dog1-3* and *dog1-4* mutants recovered (Figs 2A and B, and EV3D). To confirm this observation by other means, watering was withheld for a short time (2.5–3 days), and at this time point, there was no major visible phenotypical difference between WT and *dog1* mutant (Fig EV3E). This resulted in *DOG1* sense mRNA upregulation (Fig 2C). Subsequently, the expression of stress response marker genes *KIN1*, *RD29A*, *RD29B*, *RD22* and *RAB18* was analysed by RT–qPCR. In agreement with a previous report [48], we observed strong upregulation of all tested marker genes upon drought treatment in Col-0 (WT) plants. In contrast, upregulation of four out of the five tested genes (*KIN1*, *RD29B*, *RD29A*, *RAB18*) was diminished in the *dog1-3* mutant (Fig 2D). For example, we observed > 147-fold induction of *KIN1* in WT plants, but only ~ 34-fold upregulation in the *dog1-3* mutant in response to drought (Fig 2D). Furthermore, ABA treatment triggered similar responses in the expression of the selected marker genes (Fig EV4).

Thus, by examining the response of *asDOG1* expression to plant hormones, we discovered a novel and unexpected function of the

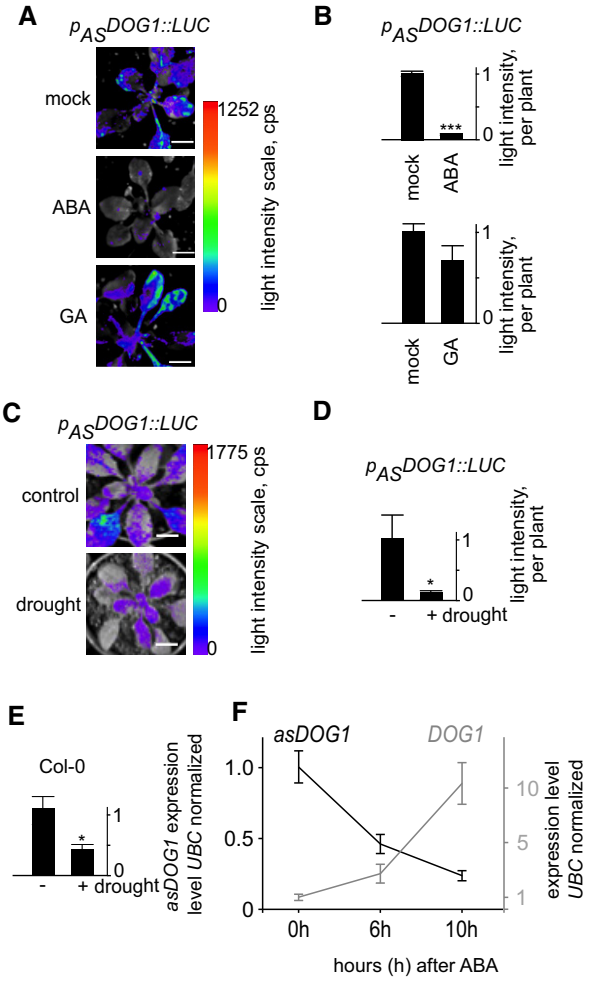

Figure 1. *DOG1* antisense expression is selectively regulated by ABA and drought in *Arabidopsis* leaves. Application of the plant hormone ABA but not GA affects luciferase activity driven by the antisense promoter.

A Representative picture of plants carrying p_{AS}*DOG1::LUC* sprayed with mock solution, ABA or GA and imaged 24 h later. The full pictures are shown in Fig EV2 and Appendix Fig S1. Scale bar, 1 cm.

B Quantification of emitted light intensity per plant after treatment with ABA and GA, compared with mock treatment.

C Antisense expression is reduced in response to drought conditions. The full pictures are shown in Fig EV3. Scale bar, 1 cm.

D Quantification of emitted light intensity per plant for control and drought-treated plants.

E Strand-specific RT–qPCR *asDOG1* expression analysis of 3-day Col-0 (WT) drought-treated vs. control plants.

F The *asDOG1* expression level (black line) is reduced while *DOG1* sense expression (grey line) is increased after application of ABA. Leaves of mature Col-0 (WT) 40-day-old plants were sprayed with ABA and collected 0, 6 and 10 h later for RNA extraction and RT–qPCR. Signals were normalized against the level of the *UBC* transcript.

Data information: The data points are the averages for at least three biological replicates and are normalized against the mock-treated value. Error bars represent standard deviation. * and *** represent *t*-test *P*-values of < 0.05 and < 0.001, respectively.

major seed dormancy QTL *DOG1* in drought response. *DOG1* mRNA expression was induced by ABA and drought (Figs 1F and 2C) and *DOG1* knockout plants showed weaker induction of stress marker

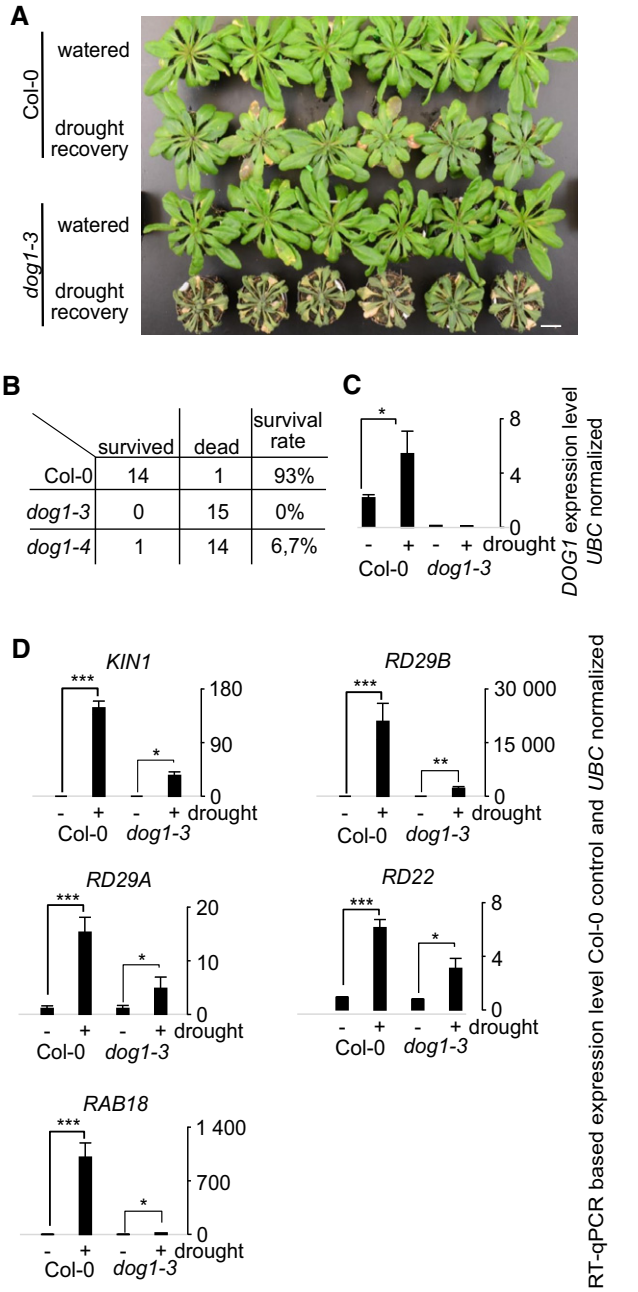

Figure 2. *dog1* mutant plants are susceptible to drought stress.

A Col-0 (WT) and *dog1-3* mutant plants were either watered normally or subjected to water withdrawal for 5 days and then watered again and allowed to recover for 2 days. A picture of a representative experiment is shown. Scale bar, 2 cm.

B Quantification of recovered and dead Col-0 (WT), *dog1-3* and *dog1-4* plants; plants were scored as dead when unable to grow and produced offspring after an extended recovery period.

C Analysis of *DOG1* sense expression in drought-treated vs. control Col-0 (WT) and *dog1-3* mutant plants, $n = 4$.

D *dog1-3* mutant plants are defective in the regulation of the majority of the tested drought marker genes. Col-0 (WT) and *dog1-3* mutant plants were subjected to drought for 3 days, and the expression of five marker genes was determined using RT–qPCR, $n = 4$.

Data information: Error bars represent standard deviation, and *, ** and *** represent *t*-test *P*-values of < 0.05, < 0.01 and < 0.001, respectively.

genes following water deprivation as well as enhanced susceptibility to drought (Fig 2 and Appendix Fig S2).

ABA-dependent *DOG1* regulation requires antisense transcription

In response to ABA and drought, we observed upregulation of *DOG1* and downregulation of *asDOG1* (Figs 1, 2, EV2 and EV3, Appendix Fig S2). To further characterize the *DOG1* response to ABA, we studied plants expressing a full-length *DOG1* transgene fused with the *LUC* reporter gene and driven under control of *DOG1* sense promoter (p_{DOG1}*LUC::DOG1*). These plants were subjected to ABA treatment at different stages of development and LUC activity measured (Fig 3A and B). This analysis showed that *DOG1* transcription was significantly increased at 10 days, but the strongest induction was observed in 40-day-old plants, in our growing conditions corresponding to the stage just before bolting. Given the ability of the antisense promoter to respond to ABA when separated from sense transcription (Fig 1A and B), we tested whether the inverse was the case for the *DOG1* sense promoter. We found that removal of *DOG1* antisense transcription rendered the truncated construct p_{DOG1}*shDOG1::LUC* insensitive to ABA at all tested stages of development (Fig 3C and D). This Δ antisense version of *DOG1* was expressed at levels similar to or higher than those observed after ABA treatment of p_{DOG1}*LUC::DOG1* plants (Figs 3C and D, and EV2C and D). Importantly, RT–qPCR analysis for sense and strand-specific analysis for antisense confirmed that *DOG1* is induced and *asDOG1* is reduced at all tested developmental stages, with the strongest effect observed at older stages (Fig 3E and F).

This indicated that the deleted 3′ region contains elements required for the ability of *DOG1* to respond to ABA and drought. The construct p_{DOG1}*shDOG1::LUC* extends to the end of *DOG1* exon 2 and lacks not only the antisense promoter but also the alternative splice sites used to generate the long three-exon version of *DOG1* mRNA, as well as the proximal and distal polyA sites [14]. RT–qPCR analysis showed that neither alternative splicing nor use of the alternative polyA sites was affected in the response to ABA (Appendix Fig S2C and D). This indicates *asDOG1* as a candidate for mediating upregulation of *DOG1* mRNA in response to ABA.

To confirm the role of *asDOG1* in *DOG1* regulation in response to drought, we took advantage of the occurrence of canonical promoter-like features in antisense promoters of *Arabidopsis* previously demonstrated by us [31]. In agreement with the reported over-representation of TATA box elements in terminators producing antisense transcripts [31], a number of such elements were detected in the *DOG1* antisense promoter. To test their significance in the regulation of *asDOG1* expression, we mutated 32 predicted TATA elements (defined using the PLACE [49] and PlantCARE [50] web tools) in the antisense promoter driving the IRES-LUC reporter gene (p_{AS}*DOG1::LUC*). These sequences were converted to TTAA or TTTA to create the construct p_{AS}*DOG1*Δ*TATA::LUC* (Appendix Fig S3). Transgenic plants with this construct produced very weak LUC activity in comparison with those transformed with the non-mutated control p_{AS}*DOG1::LUC* construct, indicating the importance of the mutated TATA boxes in *DOG1* antisense promoter function (Fig 3G). Moreover, the application of ABA strongly suppressed expression from the control construct, but had no effect on LUC activity produced by the p_{AS}*DOG1*Δ*TATA::LUC*-transformed plants (Fig EV5).

The mutation of TATA boxes in the *DOG1* antisense promoter appeared to effectively suppress its activity. Next, we introduced the TATA box mutations into the context of the genomic *DOG1* locus (p_{DOG1}*LUC::DOG1*Δ*TATA*), which allowed us to directly test the effect of antisense on *DOG1* expression and its responsiveness to ABA. The p_{DOG1}*LUC::DOG1*Δ*TATA* construct was expressed at a much higher level than the control construct p_{DOG1}*LUC::DOG1*, and it had lost the ability to respond to ABA (Fig 3H).

In summary, removal of the *DOG1* 3′ region containing the antisense promoter or mutation of TATA box elements required for *DOG1* antisense transcription rendered the *DOG1* gene unresponsive to ABA. These data are consistent with a model in which under standard conditions *DOG1* expression is continuously silenced by antisense. In response to ABA and drought, antisense promoter activity is suppressed resulting in release of silencing and upregulation of *DOG1* sense expression.

Drought resistance phenotype of plants expressing antisense-deficient *DOG1*

Our data showed that *DOG1* antisense transcription is crucial for the ABA/drought response that results in the release of antisense-mediated silencing of *DOG1* sense expression. Notably, *dog1* mutants showed enhanced sensitivity to drought (Figs 2 and EV3D), which indicated that constitutive high-level expression of *DOG1* caused by the removal of antisense expression might lead to drought resistance. To test this hypothesis, we examined transgenic lines expressing the short *DOG1::LUC* fusion construct under the control of the native promoter (p_{DOG1}*shDOG1::LUC*). This construct was shown to be functional in seeds, as judged by its ability to partially complement the *dog1-3* seed dormancy phenotype [9]. All three independent transgenic lines tested showed increased drought resistance when compared to Col-0 (WT) and transgenic plants containing p_{DOG1}*LUC::DOG1* or p_{AS}*DOG1::LUC* (Fig 4A and Appendix Fig S4). RT–qPCR analysis of selected stress marker genes showed a significant increase in the expression of four of the five tested genes (*RD29A*, *RD29B*, *RD22* and *KIN1*) in two p_{DOG1}*shDOG1::LUC* lines compared to Col-0 (WT) in the absence of ABA treatment (Fig 4B). However, following treatment with ABA, there was no clear difference in the expression of these four stress marker genes between the p_{DOG1}*shDOG1::LUC* lines and Col-0 (WT). This suggests that enhanced drought tolerance of plants with constitutive high *DOG1* expression results from partial activation of stress response in *Arabidopsis* even in the absence of drought.

Discussion

DOG1 is a novel player in the drought response of *Arabidopsis*

We have shown that *Arabidopsis dog1* mutants are sensitive to drought, while *DOG1*-overexpressing plants are more resistant to this stress than the wild type (Figs 2 and 4). Therefore, apart from its well-characterized function in seed dormancy [1,51,52], DOG1 is also an important player in the *Arabidopsis* drought response.

Interestingly, seed dormancy and drought share many similarities. Most striking is the water deficit caused by the developmental

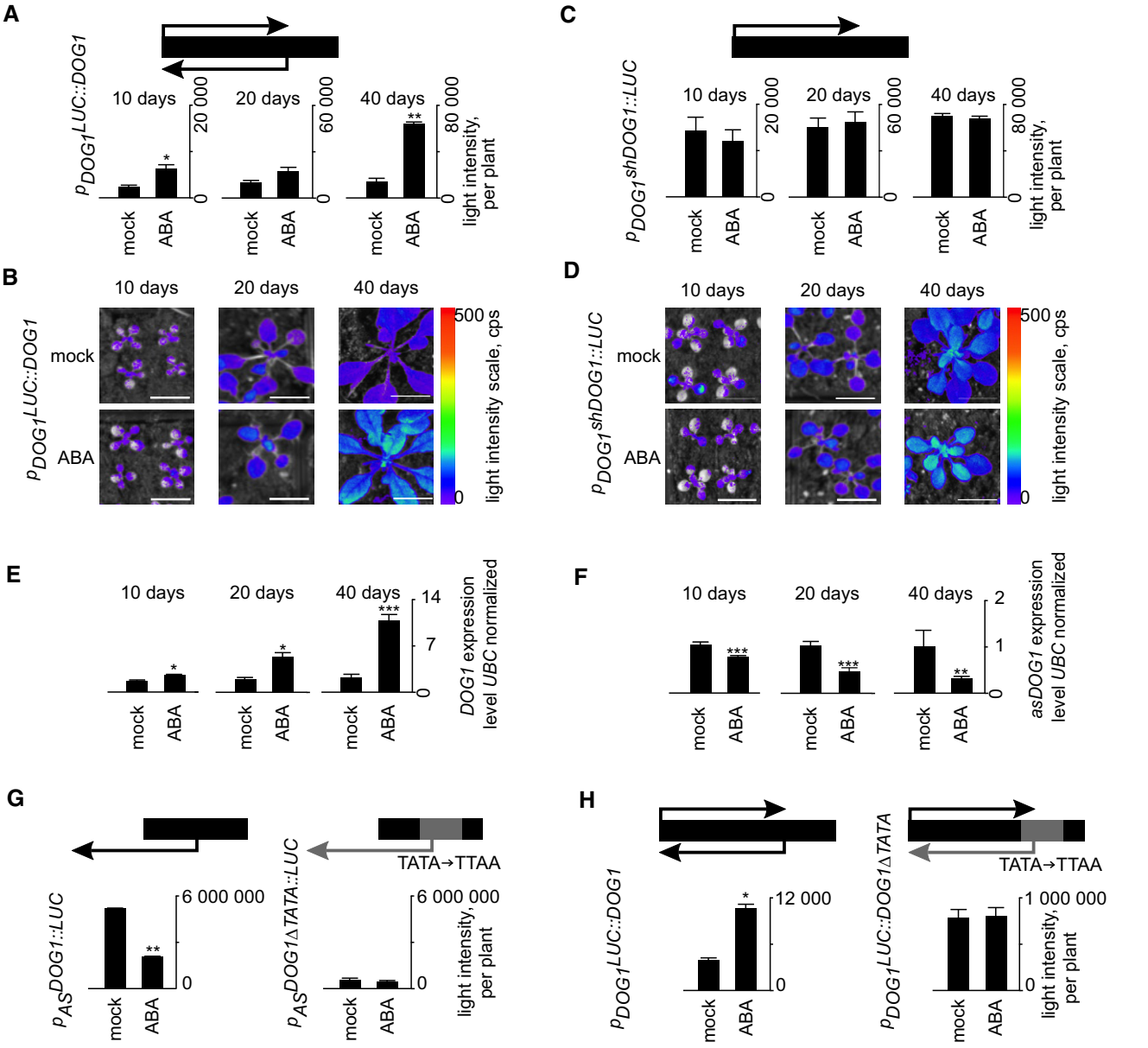

Figure 3. *DOG1* upregulation by ABA is strongest 40 days after germination and requires *asDOG1*.

A Plants expressing *LUC* fused with full-length *DOG1* (p_{DOG1}*LUC::DOG1*) were sprayed with mock solution or ABA 10, 20 and 40 days after germination and analysed 24 h after treatment. The graphs show mean emitted light intensity per plant, n = minimum 9 per each of two lines.

B Representative picture of mock- and ABA-treated p_{DOG1}*LUC::DOG1* plants. The full picture is shown in Fig EV2C and D. Scale bar, 1 cm.

C A truncated construct that lacks the *DOG1* antisense promoter region (p_{DOG1}*shDOG1::LUC*) is not induced by ABA and is highly expressed throughout development. The graphs show mean emitted light intensity per plant, n = minimum 9 per each of two lines.

D Representative picture of mock- and ABA-treated p_{DOG1}*shDOG1::LUC* plants. Scale bar: 1 cm.

E RT–qPCR analysis of *DOG1* sense mRNA level, n = 4.

F Strand-specific RT–qPCR analysis of antisense levels after 10 h of ABA treatment in 10-, 20- and 40-day-old Col-0 plants, n = 4.

G The p_{AS}*DOG1::LUC* reporter is silenced by the application of ABA, while mutation of TATA elements in the *asDOG1* promoter (p_{AS}*DOG1ΔTATA::LUC*) leads to attenuation of expression in the presence and absence of ABA, n = 8 per each of three lines.

H Mutation of TATA elements in the *asDOG1* promoter leads to high-level *DOG1* sense expression and non-responsiveness to ABA, n = minimum 8 per each of three lines.

Data information: Error bars represent standard deviation, and *, ** and *** represent t-test P-values of < 0.05, < 0.01 and < 0.001, respectively.

programme of seed desiccation and by external conditions affecting plants [53]. The similarities also extend to the molecular players involved, including the plant hormone ABA and its transduction pathway, which are required for both strong seed dormancy and drought resistance [2,54–57]. Our findings now add *DOG1* to this list. *DOG1* antisense acts as a suppressor of *DOG1* expression in seeds and also in leaves, where it suppresses seed dormancy and drought response, respectively.

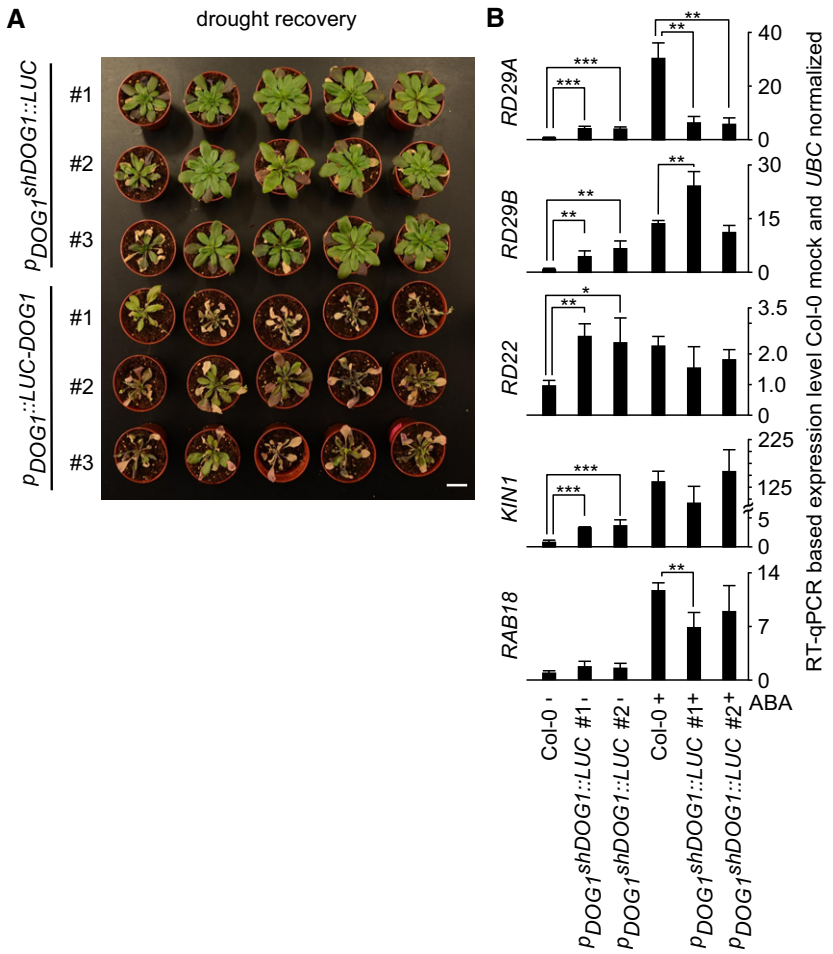

Figure 4. High-level *DOG1* expression due to antisense removal causes enhanced drought resistance in *Arabidopsis*.

A Plants expressing the truncated *asDOG1* promoter-deficient version p_{DOG1}*shDOG1::LUC* are more resistant to drought than plants expressing the full-length genomic version p_{DOG1}*::LUC-DOG1*. Three independent lines are shown for each construct, following 10 days of water deprivation. Scale bar, 2 cm.

B RT–qPCR quantification of selected drought marker genes in mock- and ABA-treated Col-0 (WT) and two independent p_{DOG1}*shDOG1::LUC* transgenic lines. Error bars represent standard deviation, and *, ** and *** represent *t*-test *P*-values of < 0.05, < 0.01 and < 0.001, respectively, $n = 4$.

DOG1 was characterized as an important player in *Arabidopsis* seed dormancy regulation over a decade ago [1]. However, despite numerous subsequent studies employing metabolomic and proteomic approaches [2,58,59], how the DOG1 protein mechanistically controls seed dormancy is still one of the most challenging questions in the field [19]. DOG1 protein is a plant-specific protein that has neither extensive homology to known proteins outside the plant kingdom nor contains any domain of known function, but its dimerization has been shown to be required for its ability to enforce seed dormancy [18].

Our analysis of stress response marker genes showed that *dog1* mutant and *DOG1*-overexpressing plants have contrasting patterns of expression for the majority of the tested genes (Figs 2D and 4B). The selected marker genes are all downstream effectors of the ABA signal transduction pathway [48], suggesting that drought resistance is mediated by DOG1 either directly or indirectly via the ABA pathway. In support of this notion, a recent study demonstrated misregulation of *ABI5*, an ABA pathway component, in *dog1* mutant seeds [2]. Genetic interaction between *DOG1* and *ABI3* or *nced9*, an ABA pathway component and ABA biosynthesis mutant, respectively, has also been reported [2,5]. Together these data suggest that DOG1 protein may control both seed dormancy and drought resistance through similar mechanisms involving the ABA pathway. We also showed that *DOG1* expression is upregulated in leaves by exogenous ABA or drought treatment (Figs 1F, 2 and 3E). This implies that *DOG1* expression is under the control of the ABA signalling pathway. It has been reported that ABA has a positive effect on expression of the *Arabidopsis DOG1 Cvi* allele [59] and *DOG1* homolog in *Lepidium sativum* and *Sisymbrium officinale* [4,60] during seed imbibition by an unknown mechanism. Recent efforts have also shown that DOG1 protein directly interacts with a number of PP2C phosphatases and genetically requires PP2C phosphatases to impose dormancy on developing seeds [61].

On the one hand, our data indicate that DOG1 acts upstream of the ABA stress response pathway, while on the other, it suggests that it is regulated by ABA. In the absence of mechanistic data concerning the function of DOG1 protein, it is currently not possible

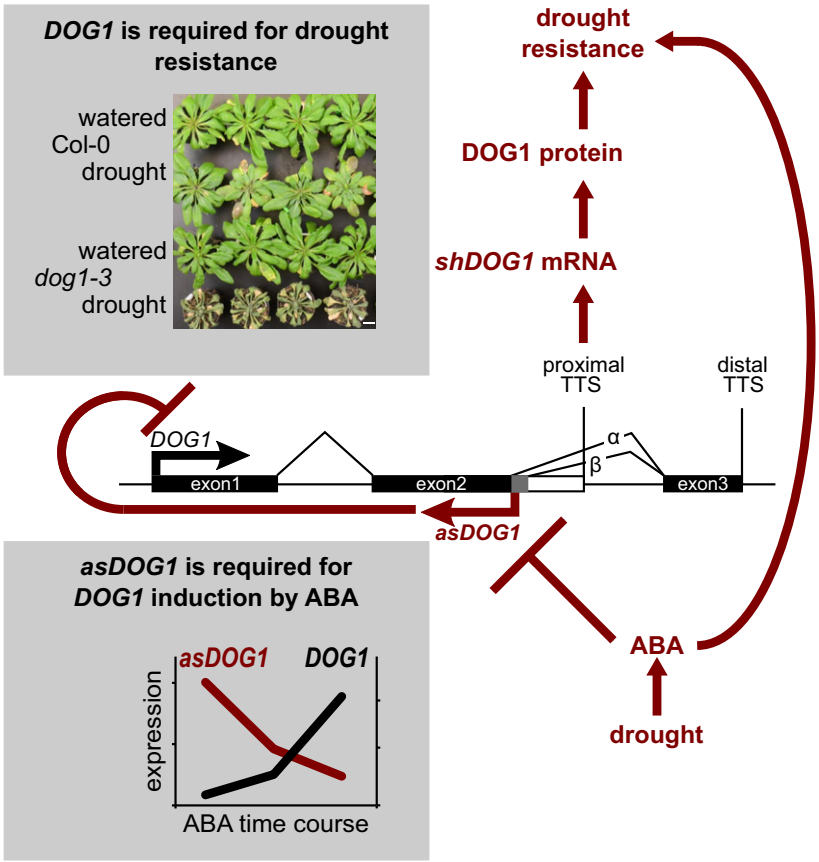

Figure 5. Model describing the drought response of *DOG1*, which depends on the function of the antisense transcript *asDOG1*.

For a detailed description see the Discussion. Drought causes ABA induction that leads to drought resistance. Part of this response is due to silencing of *asDOG1*, which results in the induction of *DOG1* transcription as shown in the bottom panel (adapted from Fig 1F). The induction of *DOG1* expression is required for and contributes to the final ABA-induced drought resistance, as shown in the top panel (adapted from Fig 2A).

to precisely place this protein within the drought response and dormancy establishment pathways.

DOG1 was initially characterized as an important player in seed dormancy regulation. In agreement with this function, *DOG1* mRNA is highly expressed in seeds but is nearly undetectable in seedlings [1,14]. Analysis of available high-throughput data show that *DOG1* expression is upregulated under osmotic stress conditions [62,63]. Despite this, *DOG1* has been repeatedly identified as a GWAS for flowering time [15], although no flowering time phenotype has been observed in *Arabidopsis dog1* mutants grown under standard conditions. However, RNAi-based silencing of the *DOG1* homolog in wheat and lettuce results in early flowering [3,5]. We have shown that mutants in *DOG1* are more susceptible to drought (Figs 2A and EV3D). Therefore, it is possible that under mild conditions of water deprivation the enhanced drought sensitivity of *dog1* mutants alters the flowering time. This remains to be tested directly, but drought has been shown to cause early flowering as part of a stress-escape response [64]. Interestingly, this effect is at least partially dependent on the ABA pathway [65,66]. In addition, recently a number of drought-associated QTLs have been identified between Kas-1 and Tsu-1, one of which maps close to *DOG1* loci [67].

Antisense requirement for the response of *DOG1* to changes in the external environment

In plants grown under standard conditions, *DOG1* mRNA is exclusively found in seeds, where its expression is extensively regulated [1]. This includes the recently described inhibition of *DOG1* expression by a long non-coding antisense transcript *asDOG1*, also known as *1GOD* [14]. Our analysis using RT–qPCR showed that *asDOG1* is highly abundant in seedlings and mature leaves, where its expression is very efficiently suppressed by drought and ABA (Figs 1, 3F, EV2 and EV4). Moreover, using multiple approaches, we demonstrated that *asDOG1* is required for the response of the *DOG1* gene to drought/ABA signals. In the absence of this antisense transcript, *DOG1* is constitutively highly expressed in leaves (Fig 3C and D). Our data define the inhibition of *asDOG1* promoter activity by ABA as an early step in the *DOG1* response to drought, which is an example of ncRNA-dependent sensing of external conditions. This observation corroborates the reported importance of ncRNA transcripts for environmental response, including *COOLAIR*, an antisense transcript generated from the *FLC* locus [32,68–70].

We recently described an antisense-based mechanism for the regulation of gene expression involving 3′ end-bound SWI/SNF

complexes [31]. Interestingly, the majority of the 3′ bound SWI/SNF targets that we identified are genes that are extensively regulated by the external and internal environment [31,71]. This suggests that the requirement for antisense of *DOG1*, in its response to environmental changes, is a common but underappreciated mechanism for regulating gene expression in plants. In agreement with this notion, multiple reports have shown that plant ncRNAs including antisense transcripts are extensively regulated by external cues [30,32,37]. Antisense transcription has been implicated in the regulation of gene expression in response to environmental cues in other eukaryotes including yeast and mammals [38,72,73]. However, in contrast to *FLC* and examples from yeast, *DOG1* antisense not only facilitates the environmental response, but seems to be absolutely required for it (Fig 3). This may reflect differences in the physiological nature of the processes controlled by antisense, and the lack of parallel pathways for the upregulation of *DOG1* in response to drought.

Based on our data, we propose a model integrating *DOG1* regulation and function in the establishment of drought resistance in *Arabidopsis* (Fig 5). In this model, the antisense transcript *asDOG1* limits *DOG1* expression in vegetative tissue. Increased ABA levels inhibit antisense expression, and the silencing of sense expression is released. The proximally polyadenylated short *DOG1* (*shDOG1*) mRNA is transcribed and translated to produce functional DOG1 protein. Based on our marker gene analysis and available genetic data from seeds, we speculate that DOG1 protein modulates the ABA pathway to implement the final level of drought resistance.

We have previously shown that in seeds, *asDOG1/1GOD* suppresses *DOG1* expression in "*cis*" but the molecular mechanism of that suppression is currently not clear. Here we show that in response to ABA and drought *asDOG1* levels are reduced, releasing *DOG1* expression. The fact that the *asDOG1* deficient lines p_{DOG1}*shDOG1::LUC* and p_{DOG1}*DOG1ΔTATA::LUC* are constitutively highly expressed in the presence and absence of ABA (Fig 3) suggests that the *asDOG1* originating from the endogenous copy is unable to silence in "*trans*" *DOG1* sense expression in leaves as shown before by us in seeds [14]. The molecular mechanism of *asDOG1*-mediated *DOG1* suppression is currently not clear. Importantly, the *DOG1* locus is devoid of DNA methylation, small RNA or high H3K9me2, suggesting that the molecular mechanism may not involve RNA interference but may be based on *cis*-acting mechanisms linked more directly to antisense transcription [74].

Our data demonstrated that *DOG1* antisense suppresses *DOG1* expression not only in seeds but also in mature *Arabidopsis* plants. By studying *asDOG1* responsiveness to stimuli, we have discovered a novel unexpected function of *DOG1* in drought response. Finally, our data showed an absolute requirement for *asDOG1* in the *DOG1* response to drought and ABA.

Materials and Methods

Plant materials, growth conditions

Arabidopsis seeds were sterilized as described [14] then plated to ½ MS plates and grown in long-day (LD) conditions at 22°C/18°C. *Arabidopsis thaliana* plants were grown in soil in a greenhouse with an LD photoperiod (16 h light/8 h dark) at 22°C/18°C. For all experiments, Col-0 was used as the WT background. The *DOG1* T-DNA insertion mutants *dog1-3* (SALK_000867) and *dog1-4* (SM_3_20886) were previously described and characterized [1,9,14]. To analyse *asDOG1* function in seeds we used the *dog1-5* mutant which has a T-DNA insertion in the *DOG1* exon 3 region, resulting in low antisense expression [14]. However, in seedlings of this mutant, the level of *asDOG1* was only slightly affected compared to Col-0 (WT) control seedlings (Appendix Fig S5), precluding its use.

Cloning of genetic constructs and generation of *Arabidopsis* transgenic plants

Several bioinformatics tools were used to predict full-length sense and antisense *DOG1* promoters by analysing the DNA sequence for the presence of potential *cis*-regulatory elements and transcription initiation sites specific for *DOG1* and surrounding genes. According to bioinformatics analysis of genomic regions, we amplified *DOG1* sense (p_{DOG1}*shDOG1::LUC*, −1,155; +1,900 from ATG) and antisense (p_{AS}*DOG1::LUC*, +1,143; −996 from TGA) promoters from *Arabidopsis* Col-0 (WT) plants and cloned into a pGWB635-LUC expression vector. The full-length *LUC::DOG1* genomic construct (p_{DOG1}*LUC::DOG1*) used in our study was previously described and characterized [14,31]. All constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain by electroporation and subsequently used for generation of stable transgenic lines, as described [75]. T3 homozygous lines were used for analysis.

RNA extraction, cDNA synthesis and adapter-mediated RT–qPCR assay

Total RNA was extracted from seedlings or leaves using TRIsure (Bio-Rad). Samples were treated with TURBO DNase (Ambion) according to the standard manufacturer protocol and efficiency of DNA removal was analysed using PCR with *PP2A* primers [14]. The quality and amount of RNA samples were tested on 1.2% agarose gel and a NanoDrop 2000 spectrophotometer; 2–2.5 μg RNA was used for cDNA synthesis (sense and antisense, correspondingly). cDNA for sense transcript was synthesized using oligo dT primers. cDNA for antisense analysis was synthesized using a gene-specific primer with an adapter, followed by qPCR with a tag-specific primer (AS_SS_RT) and *DOG1* primers (AS_F, AS_R) as described in [14]. RT–qPCR was performed using a LightCycler 480 real-time system (Roche) with SYBR Green mix (Roche). RT–qPCR results were normalized against the expression level of the *Arabidopsis UBC21* (*PEX4*) gene as described previously [76]. *P*-values presented on graphs indicate $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, calculated using a two-tailed *t*-test in Microsoft Office Excel. Error bars represent standard deviation.

Drought stress

Arabidopsis Col-0 (WT), *dog1-3* and *dog1-4* mutants and the T3 generation of *Arabidopsis* transgenic lines expressing p_{DOG1}*shDOG1::LUC*, p_{DOG1}*LUC::DOG1* and p_{AS}*DOG1::LUC* subjected to drought test were grown in soil. All seeds were imbibed at 4°C for 3 days and then planted into 7-cm pots with perlite-supplemented soil. Seven to ten days later, individual seedlings were transferred into 5-cm G-type TEKU pots (Poeppelmann, USA) which facilitate drying, and were grown for 4–5 weeks under

normal water conditions. Watering was withheld just before the bolting stage, for 4.5–5 days in the case of Col-0 (WT), *dog1-3* and *dog1-4* mutants and up to 9–10 days in case of transgenic lines expressing *DOG1* sense, antisense and full-length genomic constructs. Plants were re-watered and assessed for survival on the second day after re-watering based on the protocol adapted from [77]. Drought experiments were repeated at least three times.

Hormonal treatments and luciferase measurement

Hormonal treatment with ABA and GA_4 hormones was performed on soil-grown *Arabidopsis* seedlings or adult transgenic plants. *Arabidopsis* plants (10-, 20- and 40-day-old) were spray-inoculated with 100 μM ABA [78,79] as well as 10 μM [80] and 50 μM GA_4 (Appendix Fig S1) hormones using an EcoSprayer (www.sirchie.com, France). After spraying, plants were covered for an hour, transferred to growing chambers and subjected to either RNA extraction and RT–qPCR analysis or LUC imaging at defined time points. Luciferase treatment was performed as previously described [14]; briefly, plants were kept in darkness for 10 min, sprayed with 0.5 mM luciferin, kept in darkness for 15–20 min, and then, emitted light was measured by a NightSHADE LB985 camera, with an exposure time of 10 min. Further, LUC data were processed and calculated using IndiGo software (ver. 2.0.5.0, Berthold Technology, Germany). Light intensity units were calculated as the sum of emitted light (cps—count per second) detected by camera per plant. Error bars represent standard deviation.

Data quantification and statistics

Data were quantified as described in the relevant sections. For luminescence quantification, the light signal intensity was normalized for plant area, and error bars represent the standard deviation of the signal between individual plants. All statistical tests were done using a two-tailed *t*-test, implemented in Microsoft Office Excel.

Promoter mutagenesis

Bioinformatics analysis of *DOG1* antisense promoter revealed significant enrichment of classical TATA boxes in this region, mainly defined in the second (last) intron. We mutagenized all predicted TATA boxes in the antisense direction by introducing a point mutation in each TATA box element (TATA → TTAA or TTTA in case the former would create a new TATA box with the surrounding sequence). To mutate the identified 32 TATA boxes located in and around the second intron of the *DOG1* gene, two independent DNA fragments of the *DOG1* gene carrying corresponding mutations were synthesized by GeneArt Gene Synthesis Company (Invitrogen). Two fragments were amplified using the primers listed in Appendix Table S1, fused using fusion PCR and cloned into a pJET 1.2 vector and sequenced. Subsequently, a DNA fragment with mutations in the TATA boxes was introduced into the p_{DOG1}*LUC::DOG1* construct via BamHI-NcoI restriction sites and p_{AS}*DOG1::LUC* via EcoRI-SalI sites. Generated transgenic T2 lines were analysed for expression and for response to hormones as described above.

Expanded View for this article is available online.

Acknowledgements

Grant funding numbers: This work was funded by Polish National Science Centre grant number 2011/01/D/NZ8/03690 to SS, Polish National Science Centre grant 2011/03/B/NZ3/00297 to GD and Polish National Science Centre grant 2014/13/D/NZ3/03101 to AK.

Author contributions

RY and SS conceived the study and designed the experiments; RY and KK cloned constructs and generated the LUC-tagged *DOG1* transgenic plants; RY and KK performed LUC analysis of *DOG1* transgenic lines; RY, HF and AC performed drought experiments and RT–qPCR experiments; AK and GD were involved in analysis of drought/ABA signalling; RY and SS wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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