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RESEARCH PAPER

Arabidopsis thaliana DOF6 negatively affects germination in non-after-ripened seeds and interacts with TCP14

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Abstract

Seed dormancy prevents seeds from germinating under environmental conditions unfavourable for plant growth and development and constitutes an evolutionary advantage. Dry storage, also known as after-ripening, gradually decreases seed dormancy by mechanisms not well understood. An *Arabidopsis thaliana* DOF transcription factor gene (*DOF6*) affecting seed germination has been characterized. The transcript levels of this gene accumulate in dry seeds and decay gradually during after-ripening and also upon seed imbibition. While constitutive over-expression of *DOF6* produced aberrant growth and sterility in the plant, its over-expression induced upon seed imbibition triggered delayed germination, abscisic acid (ABA)-hypersensitive phenotypes and increased expression of the ABA biosynthetic gene *ABA1* and ABA-related stress genes. Wild-type germination and gene expression were gradually restored during seed after-ripening, despite of *DOF6*-induced over-expression. DOF6 was found to interact in a yeast two-hybrid system and *in planta* with TCP14, a previously described positive regulator of seed germination. The expression of *ABA1* and ABA-related stress genes was also enhanced in *tcp14* knock-out mutants. Taken together, these results indicate that DOF6 negatively affects seed germination and opposes TCP14 function in the regulation of a specific set of ABA-related genes.

Key words: *ABA1*, abscisic acid, after-ripening, DOF6/DOF3.2, germination, heat shock proteins, seeds, TCP14, transcription factors.

Introduction

The seed is an important organ for plant survival and species dispersion. At the end of development, mature seeds may enter a dormant state that prevents germination even under favourable conditions (Bewley, 1997), an important adaptation and a commercial trait (Gubler *et al.*, 2005). In *Arabidopsis thaliana*, as in many other species, both dormancy and germination potential are determined by the interaction between genetic and environmental factors encountered during seed development, storage, and imbibition (Finkelstein *et al.*, 2002; Donohue *et al.*, 2005; Liu *et al.*, 2005; Penfield *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; Matakiadis *et al.*, 2009; Piskurewicz *et al.*, 2009; Rodriguez-Gacio *et al.*, 2009; Yano *et al.*, 2009; Barrero *et al.*, 2010; Josse *et al.*, 2011). These processes are mediated mainly by the ratio of two antagonistic hormones: abscisic acid (ABA) and gibberellins (GAs). Dry storage gradually reduces dormancy, a process called after-ripening. Upon imbibition of after-ripened (AR) seeds, a dramatic quick decay of ABA levels is concomitant with a gradual increase in GAs, which allows germination to occur. In contrast, dormant non-germinating seeds have higher levels of ABA upon imbibition when compared to AR seeds (Ogawa *et al.*, 2003; Kushiro *et al.*, 2004; Millar *et al.*, 2006). Accordingly, genetic alterations of genes involved in ABA biosynthesis, catabolism, or signalling have a profound effect on the germination potential of seeds (Debeaujon and

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Koornneef, 2000; Lefebvre *et al.*, 2006; Penfield and King, 2009; Lee *et al.*, 2010).

The mechanism by which dormancy is regulated by after-ripening and other environmental signals is not well understood. Dry seeds contain mRNAs from the embryogenesis and maturation phases of seed development, some of them required during the early stages of germination (Holdsworth *et al.*, 2008). During after-ripening, changes in the abundance of specific mRNAs and proteins take place as a result of active transcription and translation (Bove *et al.*, 2005; Leubner-Metzger, 2005; Chibani *et al.*, 2006; Iglesias-Fernández and Matilla, 2009), although major changes in gene expression occur upon seed imbibition (Ogawa *et al.*, 2003; Yamauchi *et al.*, 2004; Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Carrera *et al.*, 2008; Kimura and Nambara, 2010). Whereas AR imbibed seeds show a large increase in RNAs encoding proteins associated with protein translation and degradation, reserve mobilization, and cell-wall modification, imbibed dormant seeds show an up-regulation of stress-related genes, many of which are expressed during seed maturation under the control of ABA signalling (Cadman *et al.*, 2006).

Transcriptomic analyses of dry and germinating seeds have shown the overlapping nature of late seed development and germination programmes. Members of several transcription factor (TF) families accumulate their mRNAs in dry seeds and play a pivotal role in triggering and maintaining gene expression during early stages of imbibition (Kimura and Nambara, 2010), or are differentially expressed between dormant and AR imbibed seeds (Yano *et al.*, 2009; Barrero *et al.*, 2010). In barley, TFs belonging to different families have been shown to regulate gene expression both during seed maturation and at early post-germinative phases and, in some cases, as occurs with the DOF TFs BPBF and HvDOF19, play opposite roles (Mena *et al.*, 1998, 2002; Moreno-Risueño *et al.*, 2007a). In the *A. thaliana* genome, the DOF family is composed of 36 different members, mainly associated with plant-specific phenomena (Yanagisawa, 2002; Lijavetzky *et al.*, 2003; Moreno-Risueño *et al.*, 2007b). Two of them, DAG1 and DAG2 were shown to influence, with opposite effects, the germination of *Arabidopsis* seeds (Papi *et al.*, 2000; Gualberti *et al.*, 2002; Gabriele *et al.*, 2010).

An *A. thaliana* DOF TF (At3g45610; DOF3.2/DOF6; Yanagisawa, 2002; Moreno-Risueño *et al.*, 2007b), hereafter DOF6, has been characterized as a negative regulator of seed germination. *DOF6* transcripts accumulate in dry seeds and decay gradually upon imbibition and during after-ripening. While constitutive over-expression of *DOF6* produced aberrant growth and sterility in the plant, inducible over-expression (IOEX) of *DOF6* upon seed imbibition triggered delayed germination, ABA-hypersensitive phenotypes, and increased expression of genes involved in ABA biosynthesis and stress. These altered responses were under the control of the AR status of the seed. A search for DOF6 protein partners identified TCP14, a TF previously identified as a positive regulator of germination potential (Tatematsu *et al.*, 2008). The same ABA-related genes were up-regulated in *ioexDOF6* and *tcp14* knock-out (KO) mutants. These results indicate that DOF6 negatively affects seed germination and that DOF6 and TCP14 play opposite roles on this process.

Materials and methods

Plant material and growth conditions

A. thaliana Columbia accession (Col-0) was used as the wild type (WT) in this study. A *dof6-1* single mutant (SALK_010732) was identified in the Salk T-DNA insertion database (http://signal.salk.edu/cgi-bin/tdnaexpress; Alonso *et al.*, 2003). *tcp14-1* and *tcp14-3* mutants were previously described in Tatematsu *et al.* (2008). *dof6-1* and *tcp14-3* mutant seeds were obtained from NASC (University of Nottingam, UK) and *tcp14-1* was provided by Prof. Lucia Colombo (Università di Milano, Italy). Plants were grown either on Petri dishes containing half-strength Murashige and Skoog (MS) medium buffered with 2 mM MES (2-*N*-morpholino ethanesulfonic acid), pH 5.7, and 0.7% (w/v) agar, or in soil, and grown to maturity at 16 h light at 22 °C /8 h dark at 20 °C and 60% relative humidity. Seeds were harvested when plants had ceased flowering and siliques were starting to dehisce and stored in the dark at 22 °C and 30% relative humidity.

Generation of transgenic lines

The *pDOF6::GUS* plasmid was generated using oligonucleotides *pDOF6-fw* and *pDOF6-rv* (Supplementary Table S1, available at *JXB* online) to amplify a 1.2 kb fragment containing the putative promoter region of *DOF6* from *A. thaliana* genomic DNA. The amplified fragment was digested with *Sal*I and *Bam*HI, cloned into the pENTR3C vector (Invitrogen, USA) and transferred by Gateway LR recombination (Invitrogen) into the destination vector pMDC163, containing the *β-glucuronidase* (GUS) reporter gene (Curtis and Grossniklaus, 2003). The *amiRNA DOF6* construct was designed using the resource at www.weigelworld.org and the oligonucleotides used were *DOF6-I-miR-fw*, *DOF6-II-miR-rv*, *DOF6-III-miR*-fw*, and *DOF6-IV-miR*-rv* (Supplementary Table S1). Cloning procedure was as described in www.weigelworld.org and the amiRNA sequence was digested with *Xho*I and *Spe*I and cloned into the pER8 vector (Zuo *et al.*, 2000). A *RNAi DOF6* construct (CATMA3a38625) from the Agrikola collection, together with the pSOUP vector, was provided by NASC (Hilson *et al.*, 2004). The *p35S::DOF6* and *ioexDOF6* constructs were generated using the oligonucleotides *DOF6-fw* and *DOF6-rv* (Supplementary Table S1) to amplify the *DOF6* open reading frame (ORF) from *A. thaliana* cDNA. The amplified fragment was digested with *Sal*I and *Eco*RI, and cloned into the pENTR3C vector (Invitrogen) and introduced by LR Gateway reaction into the destination vectors pEarleyGate201 (Earley *et al.*, 2006) and pMDC7 (Curtis and Grossniklaus, 2003). All constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 GV3101 by electroporation (in the case of *RNAi DOF6*, together with pSOUP vector) and transformed into *Arabidopsis* by the floral dip method (Clough and Bent, 1998).

Seed germination assays

WT and mutant seeds were collected at the same time and obtained from plants grown in the same conditions. Storage conditions were 22 °C and 30% relative humidity in the dark for 1 week for freshly harvested (FH) seeds and for 3 months for AR seeds. For each genotype, approximately 50 seeds were placed onto filter papers (Whatman No. 3, UK) moistened with 3 ml of sterile water in 6 cm diameter Petri dishes. Plates were sealed with Micropore tape (Micropore 3M, USA) and incubated at 22 °C under 16/8 h light/dark conditions. Germination was scored as radicle emergence through the endosperm and testa every 24 h. All germination assays were carried out in triplicate with at least two independent seed batches.

estradiol treatments

To induce gene expression in *ioexDOF6* and *amiDOF6* lines in germination assays, 17-β-estradiol (Sigma Aldrich, Spain) was diluted directly in the imbibition water to a final concentration of 50 μM. For continuous induction on agar plates, drops of 50 μM estradiol were directly added to each plant every 2 days. For induction on rosette leaves, 50 μM estradiol containing 0.02% (v/v) Tween 20 was sprayed on the leaves and samples were harvested after 16 h.

Gene expression analyses

Total RNA was isolated from seeds and other tissues, as described by Oñate-Sánchez and Vicente-Carbajosa (2008). Seeds (15 mg) from each genotype were germinated on moistened filter paper and samples were taken at different hours after imbibition (hai). The Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) was used to synthesize cDNA. For quantitative reverse transcription (RT)-PCR, 8–16 ng cDNA was used as template together with 0.5 μM of forward and reverse specific oligonucleotides (Supplementary Table S1) and Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Cycling conditions (ABI Prism 7300; Applied Biosystems) were as follows: 10 min at 95 °C and 50 cycles of 15 s at 95 °C and 60 s at 60 °C, linked to a default dissociation stage programme to detect non-specific amplification. Three technical and at least two biological replicates were included in every experiment and the ubiquitin gene *UBC21* (At5g25760) was used to normalize expression levels. Therefore, gene-specific mRNA levels shown in all figures except Fig. 5 are relative to *UBC21*.

ABA measurements

Seeds were collected 48 hai in 50 μM estradiol and processed as described in Durgbanshi *et al.* (2005).

In situ hybridization and histochemical GUS analyses

In situ hybridization was performed as described by Iglesias-Fernández *et al.* (2011). Specific oligonucleotides (*DOF6-insitu-fw* and *DOF6-insitu-rv*; Supplementary Table S1) were used to amplify a 200 bp fragment from the *DOF6* 3′-non-coding region. Sense and antisense digoxigenin (DIG)-labelled RNA probes were synthesized with the DIG RNA labelling mix (Roche Diagnostics). Probes were hybridized at 52 °C overnight.

GUS staining was performed using the protocol described by Jefferson *et al.* (1987). After 3 h of seed imbibition, testa and endosperm were separated from the embryos and were incubated with the staining solution at 37 °C until blue colour was visible. Analyses were performed using at least eight independent lines.

Yeast two-hybrid assays

A *BD-DOF6* construct was obtained by the LR Gateway recombination reaction between the entry vector pENTR3C, harbouring the *DOF6*-ORF cassette, and the destination vector pDEST32 (Invitrogen) and was transformed into the *Saccharomyces cerevisiae* pJ694α strain (James *et al.*, 1996). Yeast two-hybrid screening of an arrayed TF yeast library containing c. 1200 *A. thaliana* TFs was performed as described by Castrillo *et al.* (2011). The plates used in the screening to select the positive interactions contained 30 mM 3-amino-1,2,4-triazole (3-AT; Sigma Aldrich). Yeast transformation was done by the polyethylene glycol method. Quantification of β-galactosidase (LacZ) activity in liquid culture in the *S. cerevisiae* SFY526 strain was calculated using Miller's formula as described by Lara *et al.* (2003).

Bimolecular fluorescent complementation

TCP14 ORF was amplified from *A. thaliana* cDNA using the oligonucleotides *TCP14-GW-fw* and *TCP14-GW-rv* (Supplementary Table S1) and cloned into the pDONR221 plasmid by the BP Gateway reaction. Both *DOF6* and *TCP14* were fused in frame with the N- and C-terminal fragments of yellow fluorescent protein (YFP), respectively, by LR Gateway recombination with the destination vectors pE-SPYNE-GW and pE-SPYCE-GW (Weltmeier *et al.*, 2006). Co-bombardment experiments were done in inner epidermal layers of fresh onions (*Allium cepa*) using a biolistic Helium gun device (DuPont PDS-1000; BioRad Laboratories, Hercules, CA, USA). The fluorescence emission was observed after 24 h of incubation at 22 °C in the dark under a fluorescence Zeiss Axiophot microscope (Carl Zeiss, Germany) with the following filter set: excitation, 450–490 nm; emission, 520 nm. Images were captured with a CCD colour Leica DFC300FX camera and processed with the Leica Application Suite 2.8.1 build 1554 software (Leica, http://www.leica.com). Each bombardment was performed in four independent plates and complementation was confirmed in two independent assays. Transformation efficiencies were estimated by bombardment with a *p35S::GFP* construct.

Results

DOF6 *is expressed in A. thaliana seeds*

Since several DOF proteins have been strongly linked to the regulation of gene expression during seed development, it was decided to explore whether other phylogenetically related DOFs could have a regulatory role in this organ. *DOF6* was selected for further studies since quantitative RT-PCR analyses revealed that its mRNA was preferentially expressed in siliques, although it was also found in leaves, roots, and flowers (Fig. 1A). During silique development, *DOF6* was expressed throughout the maturation phase, reaching its maximum expression levels in dry seeds (Fig. 1B). Upon imbibition of FH seeds, *DOF6* mRNA levels gradually decreased, being 20 times less abundant at 48 hai than in dry seeds (Fig. 1C). In order to localize the expression of *DOF6* in seeds, *A. thaliana* Col-0 plants were transformed with the GUS reporter gene under the control of the *DOF6* promoter. GUS activity was detected in the vascular tissues of the embryo at 3 hai, an expression pattern also observed at 24 hai (Fig. 1D). To confirm this pattern, mRNA *in situ* hybridization experiments were performed in seeds imbibed for 24 h. As shown in Fig. 1E, *DOF6* transcripts were observed in the embryo, mainly in the vascular tissues, and could not be detected in the samples hybridized with a control sense probe (Fig. 1F).

DOF6 *over-expression causes severe growth defects in* Arabidopsis

To analyse *DOF6* function in the plant, T-DNA insertion mutants were searched in public *A. thaliana* KO collections. Only one line was found to contain an insertion in the *DOF6* genomic region (SALK_010732). Homozygous plants for this insertion line did not show alterations in *DOF6* expression levels, probably due to the position of the T-DNA located at 900 bp upstream of the initiation codon (Supplementary Fig. S1). Therefore, to reduce *DOF6*

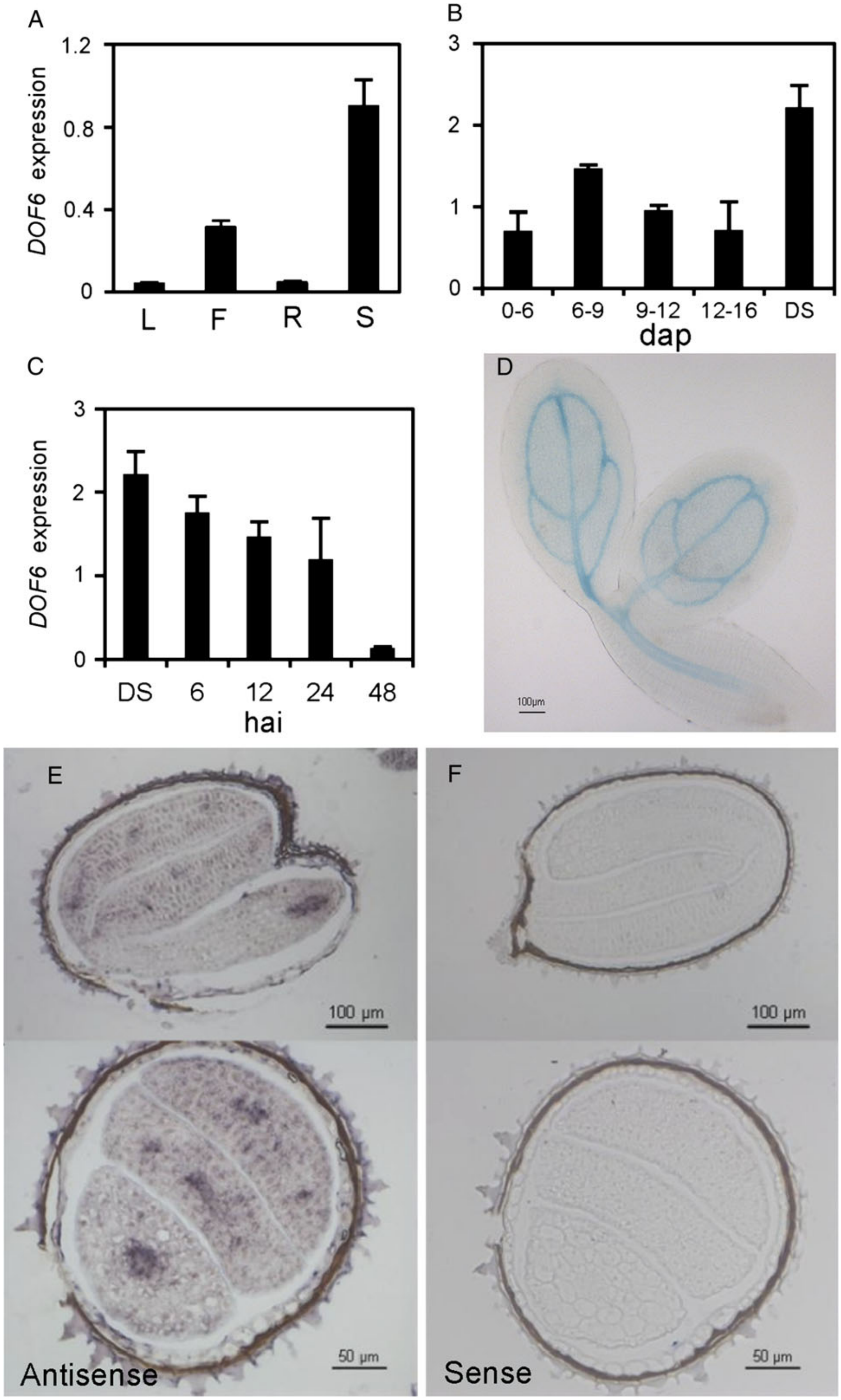

Fig. 1. *DOF6* expression patterns*. DOF6* mRNA levels relative to *UBC21* in: (A) wild-type plants in different plant organs (L, adult rosette leaves; F, flowers; R, roots; S, pool of developing siliques from 4 to 15 days after pollination (dap); DS, dry seeds); (B) siliques at different dap and (C) seeds at different hours after imbibition (hai). Means and standard errors for three replicates are shown. (D) Expression of the GUS reporter gene driven by the *DOF6* promoter in seeds imbibed for 3 h. Seed covers were removed before incubating with the staining solution. (E, F) *In situ* hybridization of *DOF6* transcripts in Col-0 seed sections. Seeds were imbibed for 24 h and hybridized using antisense (E) and sense (F) *DOF6* probes. Gene-specific mRNA levels are relative to *UBC21*. Bars, 100 µm (D), 100 and 50 µm (E and F).

mRNA levels, transgenic plants carrying constructs for constitutive or inducible expression of *DOF6*-specific ihpRNAs or amiRNAs, respectively, were generated. *DOF6* mRNA levels were not reduced in any of the 15 ihpRNA or eight amiRNA transgenic lines analysed (Supplementary Fig. S1).

To study the effect of *DOF6* over-expression, *Arabidopsis* plants were transformed with the *DOF6* ORF under the control of the CaMV 35S promoter (*p35S::DOF6*). Eight independent transgenic lines were selected with higher *DOF6* mRNA levels than the WT. The expression of *DOF6* in three of these lines is shown in Fig. 2A. All these lines showed a stunted phenotype and severe growth defects (Fig. 2B). Only one line was able to develop flowers, although producing very few non-viable seeds (Fig. 2C; Supplementary Fig. S2).

Since constitutive over-expression of *DOF6* mRNA levels had deleterious effects to the plant, an IOEX strategy was adopted. *Arabidopsis* plants were transformed with the *DOF6* ORF under the control of an estradiol-inducible promoter (Curtis and Grossniklaus, 2003). The transgenic lines obtained, hereafter *ioexDOF6*, increased *DOF6* mRNA expression after 16 h of being sprayed with 50 μM estradiol, while no significant differences with WT levels were observed when sprayed with a mock solution (Fig. 2D). *ioexDOF6* plants developed normally when grown in the absence of estradiol but, when the induction was maintained by adding fresh estradiol to the growing media every 2 days, seedling growth was delayed, producing a stunted phenotype similar to that observed for the constitutive over-expression lines (Fig. 2E and 2F).

DOF6 *over-expression delays seed germination in freshly harvested but not in after-ripened seeds*

To study the effect of *DOF6* over-expression on germination, FH *ioexDOF6* seeds were imbibed in the presence or absence of 50 μM estradiol. As shown in Fig. 3A, *DOF6* mRNA levels were similar in *ioexDOF6* and in WT dry seeds, indicating the absence of significant leaky expression from the inducible promoter. Upon imbibition with estradiol, *DOF6* mRNA levels increased up to 30 times in *ioexDOF6* seeds. The germination ability of WT seeds was similar in the absence or presence of estradiol and undistinguishable from that observed for the *ioexDOF6* lines in the absence of the inducer (Fig. 3B and 3C). However, in the presence of estradiol, FH *ioexDOF6* seeds showed delayed germination when compared to WT seeds (Fig. 3C). Although the induction of *DOF6* mRNA levels varied between three selected *ioexDOF6* lines, and even between descendants of the same plant, the delayed germination phenotype was consistently observed in the three independent transgenic lines, and two showing lower germination percentages were chosen for further studies (Supplementary Fig. S3).

To assess if this delayed germination phenotype is influenced by the after-ripening status of the seed, a similar germination experiment was done with AR seeds (3 months of storage at 22 °C; Fig. 3D). In this case, the germination kinetics were identical in WT and in *ioexDOF6* seeds in the presence of estradiol. When the experiment was repeated with seeds that had been after-ripened for different periods, the delayed germination of FH *ioexDOF6* seeds disappeared gradually with the dry storage time (Fig. 3E). Suppression of the delayed phenotype was also observed in FH *ioexDOF6* seeds when stratified (4 °C, 48 h) or imbibed at 17°C (Supplementary Fig. S4). To investigate whether *DOF6* endogenous transcripts were being affected by after-ripening, *DOF6* mRNA levels were quantified in dry and in imbibed FH and AR seeds. While *DOF6* mRNA levels were similar at 24 hai in FH and AR seeds, FH dry seeds contained nearly ten times more transcripts than AR dry seeds (Fig. 3F). These results indicate that the *ioexDOF6*-delayed germination phenotype is influenced by the after-ripening and dormancy status of the seed and that *DOF6* mRNA levels decay during after-ripening and upon imbibition of FH seeds.

DOF6 *over-expression increases* ABA1 *mRNA in freshly harvested seeds*

Since *DOF6* mRNA levels in seeds decreased during after-ripening and upon seed imbibition and increased during seed maturation, a pattern that resembled ABA dynamics in seeds (Kushiro *et al.*, 2004; Vicente-Carbajosa and Carbonero, 2005; Millar *et al.*, 2006), it seemed appropriate to quantify *DOF6* mRNA levels in seeds in response to ABA. FH and AR WT seeds were imbibed in the presence or absence of ABA, and *DOF6* transcripts were quantified during seed imbibition. As shown in Fig. 4A and B, *DOF6* had different expression patterns in FH and AR imbibed seeds, both with or without ABA during the first hours of imbibition, but the transcript levels were not significantly altered by the presence of ABA after 24 h.

To test the ABA effects on the *ioexDOF6* germination phenotype, FH and AR *ioexDOF6* and WT seeds were imbibed with estradiol in the absence or presence of ABA and their germination scored. When FH seeds were used, the germination delay of *ioexDOF6* seeds compared to WT was enhanced by the presence of ABA and an overall reduction in germination efficiency was detected for all the genotypes tested (Fig. 4C). The AR *ioexDOF6* seeds, which had the same germination kinetics as the WT in the absence of ABA, showed a delayed germination in its presence (Fig. 4D). These data indicate that the *ioexDOF6* lines are hypersensitive to exogenous ABA both in FH and in AR seeds.

FH *ioexDOF6* seeds germinated in the presence of estradiol were used to quantify mRNA levels of key genes involved in ABA metabolism and signalling pathways: *ABA1*, *NCED6*, and *NCED9*, encoding a zeaxanthin epoxidase and two 9-*cis*-epoxycarotenoid dioxygenases, respectively, involved in ABA biosynthesis (Frey *et al.*, 1999; Audran *et al.*, 2001; Lefebvre *et al.*, 2006); *CYP707A2*, encoding an ABA 8′-hydroxylase involved in ABA degradation (Okamoto *et al.*, 2006); and *ABI3* and *ABI5,* encoding TFs involved in ABA signalling (Giraudat *et al.*,

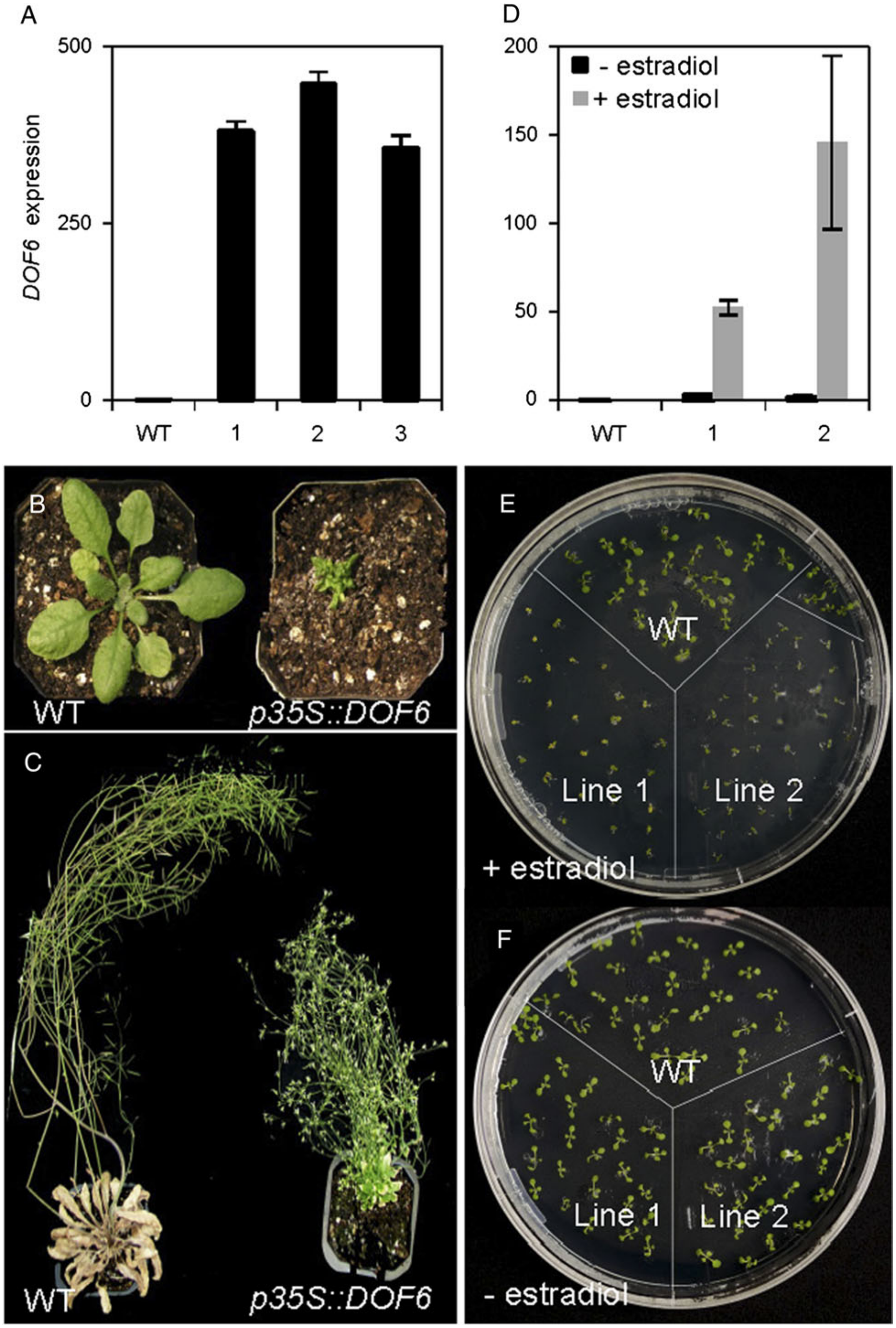

Fig. 2. *DOF6* over-expression produces growth and developmental defects on the plant. (A) *DOF6* mRNA levels in rosette leaves of wild-type (WT) and *p35S::DOF6* transgenic plants. Representative independent transgenic lines are shown. (B and C) Three- and eight-week-old plants over-expressing *DOF6* (*p35S::DOF6*), respectively and show a dwarf phenotype compared to the WT and are unable to produce seeds. (D) *DOF6* mRNA levels in WT and two representative *ioexDOF6* lines, before and 16 h after spraying with 50 µM estradiol. (E and F) WT and two representative *ioexDOF6* lines germinated on half-strength Murashige and Skoog agar with 50 µM estradiol added every 2 days (E) and without estradiol (F); pictures were taken 3 weeks after sowing. Gene-specific mRNA levels are relative to *UBC21*.

1992; Finkelstein and Lynch, 2000; Lopez-Molina *et al.*, 2002). *ABA1* was significantly induced in *ioexDOF6* lines compared to the WT (more than three times in one line and more than five in the other; Fig. 5A). Such differences were not found in the expression levels of *NCED6*, *NCED9*, *CYP707A2*, *ABI3*, and *ABI5* between the WT and the *ioexDOF6* lines, although *NCED9* and *ABI5* were induced more than two-fold in one of the *ioexDOF6* lines (Fig. 5A). Then, the expression of seed ABA-inducible genes was analysed, such as those encoding late embryogenesis

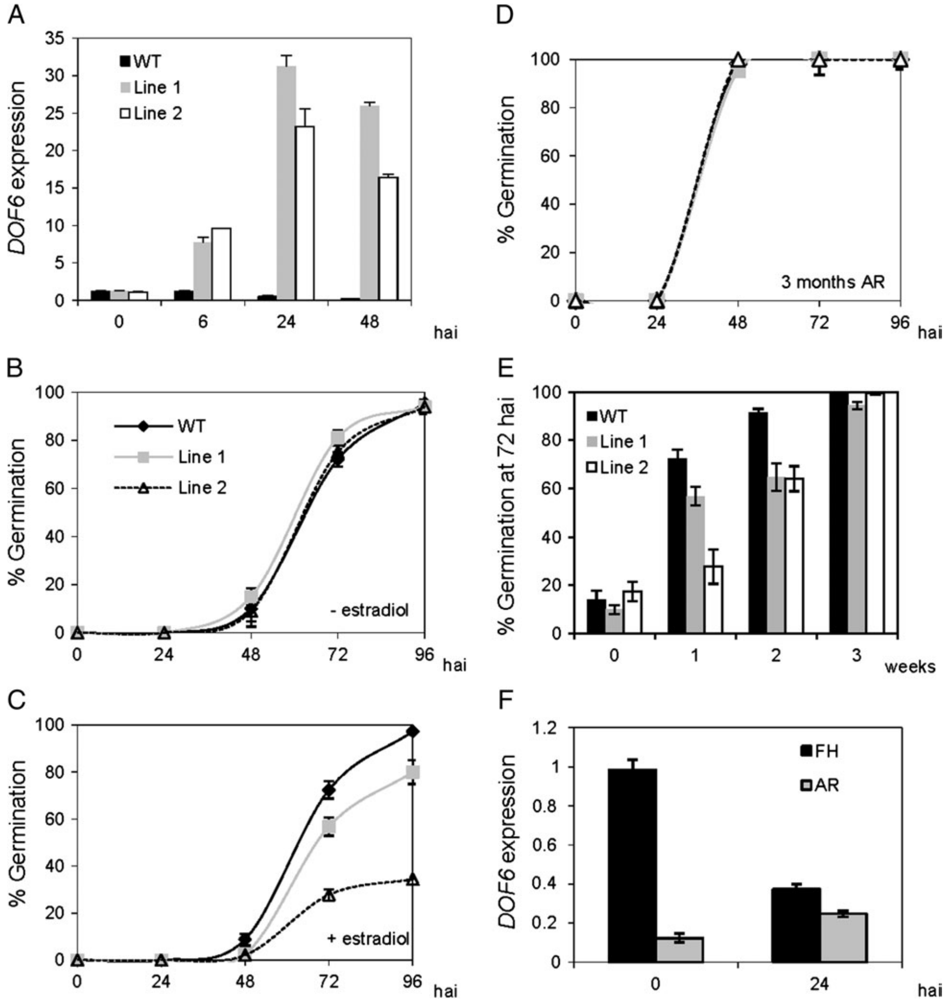

Fig. 3. Germination of *ioexDOF6* seeds and *DOF6* expression in freshly harvested (FH) and after-ripened (AR) seeds. (A) *DOF6* expression levels in FH wild type (WT) and two representative *ioexDOF6* lines upon imbibition in 50 μM estradiol. (B and C) Germination of FH WT and *ioexDOF6* seeds imbibed in 50 μM estradiol (C) and controls without estradiol (B). (D) Germination of AR WT and *ioexDOF6* seeds in 50 μM estradiol. (E) Germination percentage of WT and *ioexDOF6* seeds at 72 hours after imbibition (hai) in 50 μM estradiol after different weeks of dry storage. (F) *DOF6* mRNA levels in FH and in AR WT dry seeds and 24 hai. Gene-specific mRNA levels are relative to *UBC21*.

abundant (LEA) and small heat shock proteins (sHSPs; Kotak *et al.*, 2007; Holdsworth *et al.*, 2008). It was found that s*HSP17.4* and s*HSP22* were induced up to ten times in the *ioexDOF6* lines compared with the WT (Fig. 5B). None of these genes showed an increased expression when AR seeds were used for the analyses (data not shown).

DOF6 interacts with TCP14, a transcription factor that regulates embryonic growth potential during seed germination.

Since transcriptional regulation is a combinatorial process, possible interactors of DOF6 were looked for. To identify such interacting proteins, a yeast two-hybrid screening was performed, using as prey an arrayed library of c. 1200 *A. thaliana* TFs fused to the GAL4 activation domain (*AD-TF*). The *DOF6* ORF was fused to the GAL4 DNA-binding domain (*BD-DOF6*) and used as a bait against each of the library TFs by a high-throughput mating assay (Castrillo *et al.*, 2011). A positive interaction with TCP14 was identified and confirmed by growing the mated cells on the appropriate auxotrophic media with increasing concentrations of 3-AT (Fig. 6A). This interaction was quantified using a different yeast strain (*S. cerevisiae* SFY526) that contains a *LacZ* reporter gene under the control of GAL4BD binding sites. The SFY526 strain was transformed with *BD-DOF6* and *AD-TCP14* or with *BD-DOF6* and *AD-TCP1* constructs. TCP1 was used as a negative control since it is a member of the TCP family that did not interact with DOF6 in the screening. The LacZ activity of the yeast strain carrying the *BD-DOF6* and *AD-TCP14* constructs was several orders of magnitude above that of the control, indicating that this is a strong interaction (Fig. 6B). Moreover, these results confirm that this interaction is independent of yeast ploidy or genotype.

To validate the DOF6–TCP14 interaction *in planta*, bimolecular fluorescent complementation experiments were carried out. *DOF6* and *TCP14* ORFs were translationally

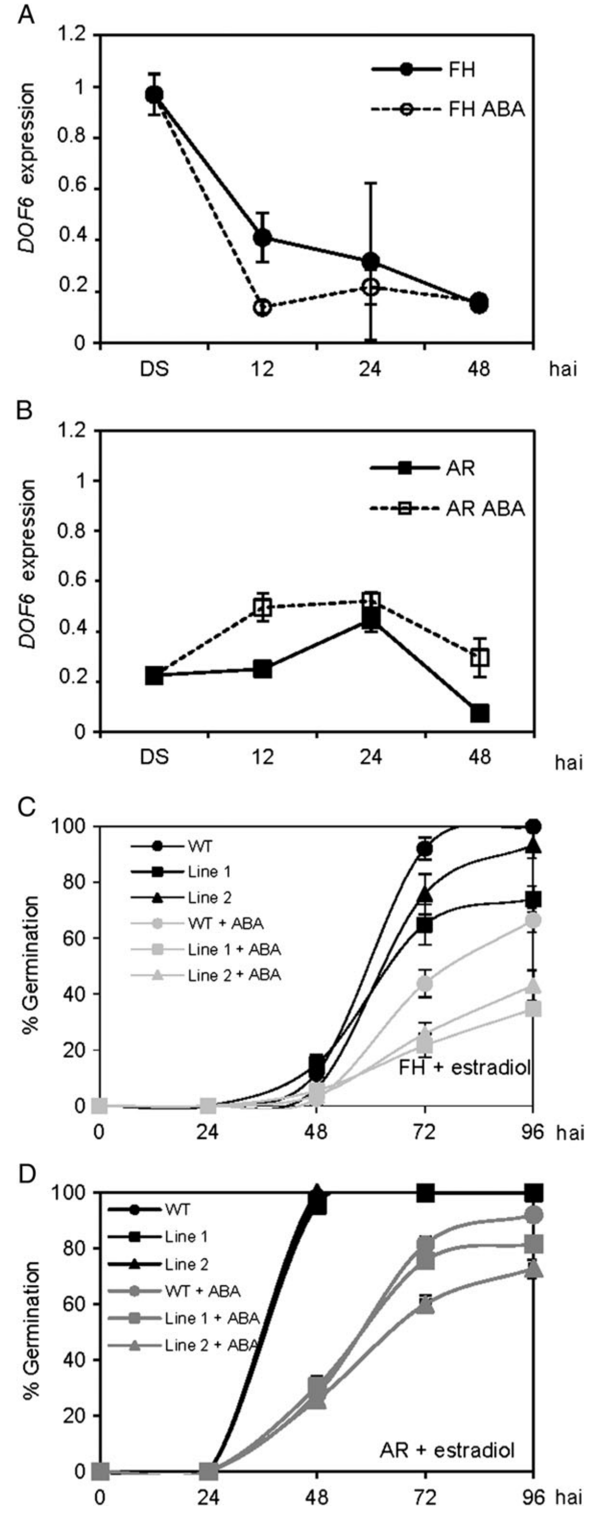

Fig. 4. ABA effect on *DOF6* expression and *ioexDOF6* seed germination. (A and B) *DOF6* expression by quantitative RT-PCR in freshly harvested (FH; A) and after-ripened (AR; B) Col-0 seeds imbibed in water with 2 and 5 μM ABA, respectively. (C and D) Germination of FH (C) and AR (D) wild-type (WT) and *ioexDOF6* seeds in water supplemented with 50 μM estradiol and 0.5 and 1 μM ABA, respectively. Germinating percentages are represented as the mean±standard error from three replicates. Germination patterns were confirmed in two different seed batches. Gene-specific mRNA levels are relative to *UBC21*.

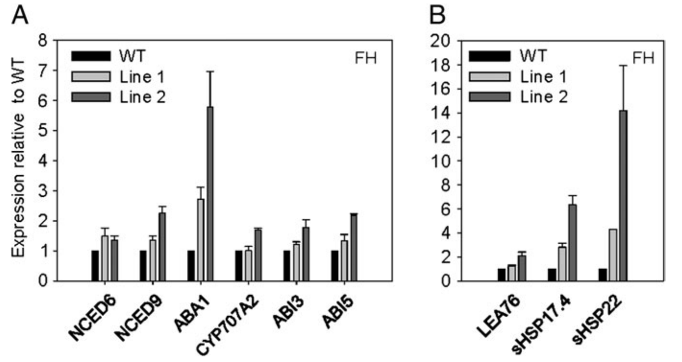

Fig. 5. mRNA levels of ABA-related genes in germinating freshly harvested (FH) *ioexDOF6* seeds. Expression of genes involved in ABA metabolism and signalling (A) and ABA-inducible genes (B) was compared by quantitative RT-PCR between wild-type (WT) and *ioexDOF6* FH seeds 48 hours after imbibition in 50 μM estradiol. Gene-specific mRNA levels are relative to UBC21. All the *UBC21* normalized expression values are relative to the values obtained for each gene in the WT sample.

fused to the N- and C-terminal fragments of YFP, respectively, and these constructs were used for transient expression by bombardment of epidermal onion cells. When *N-YFP-DOF6* and *C-YFP-TCP14* constructs were co-bombarded, YFP fluorescence was observed in the nucleus, indicating that DOF6 and TCP14 proteins interact *in planta* (Fig. 6C). As expected, no reconstruction of YFP activity was achieved when different combinations of TF and/or empty vectors were co-bombarded (data not shown).

DOF6 and TCP14 participate in the regulation of ABA1 *and* sHSP *genes in imbibed seeds*

TCP14 had been previously described as a positive regulator of embryonic growth potential during seed germination (Tatematsu *et al.*, 2008) and the phenotypes observed for *tcp14* KO seeds resembled those of *ioexDOF6* seeds. To test if DOF6 and TCP14 could be involved in the regulation of a common set of genes, the expression of those genes that were found to be induced more than three times during FH seed imbibition in the *ioexDOF6* lines were quantified in FH seeds from two previously described *tcp14* KO lines (*tcp14-1* and *tcp14-3*; Tatematsu *et al.* 2008). While the mRNA levels of *TCP14* were reduced in both KO lines when compared to the WT, as expected, the expression of *ABA1*, s*HSP17.4*, and s*HSP22* was enhanced (Fig. 7A), as occurs in the *ioexDOF6* lines. These results indicate that DOF6 and TCP14 have opposite effects (activator and repressor, respectively) on the expression of *ABA1*, *sHSP17.4*, and *sHSP22*.

To analyse a possible cross-regulation between DOF6 and TCP14, their mRNA levels in *tcp14* KO and *ioexDOF6* germinating seeds, respectively, were quantified. As shown in Fig. 7B, no differences in mRNA levels were detected between the WT and mutant plants, indicating that DOF6 or TCP14 are not regulating each other's expression.

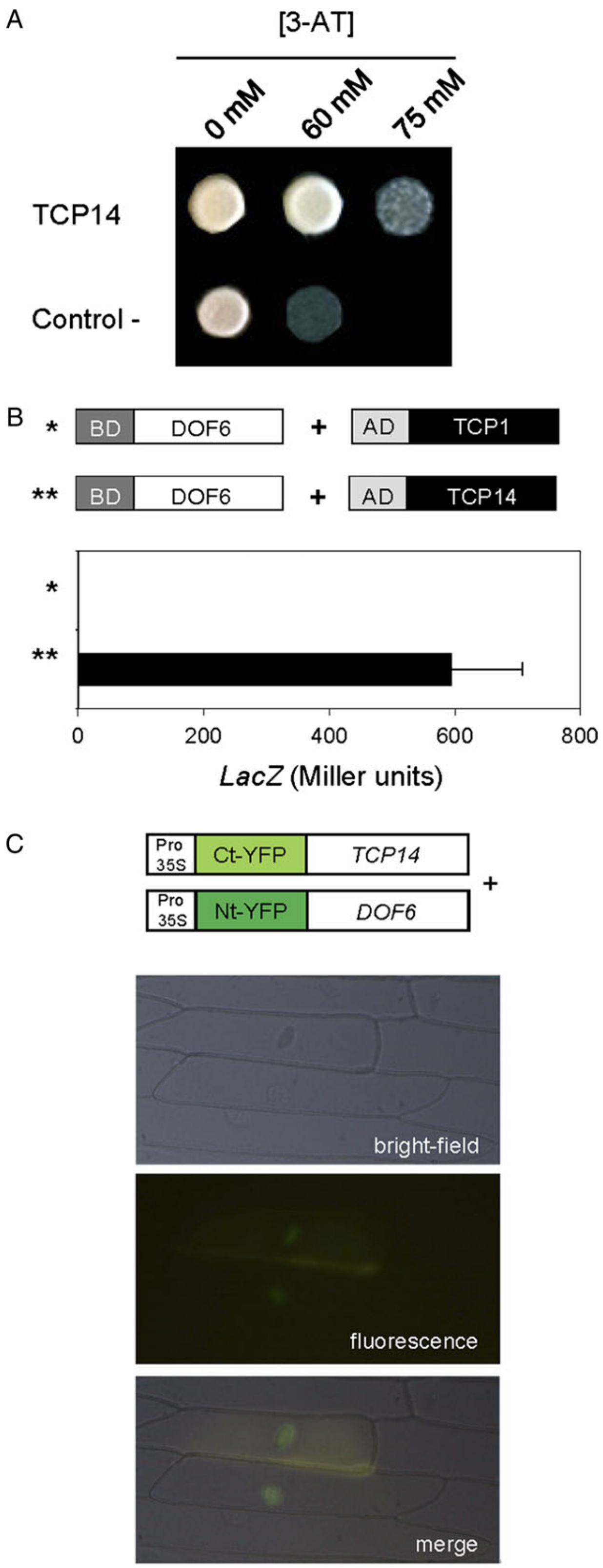

Fig. 6. DOF6 interaction with TCP14. (A) Diploid cells carrying *BD-DOF6* and *AD-TCP14* (TCP14) or *BD-DOF6* and AD empty vector (control –) were grown on the appropriate selection media with increasing concentrations of 3-amino-1,2,4-triazole (3-AT). (B) Quantification of the DOF6–TCP14 interaction in yeast using the *β-galactosidase* reporter gene. An *AD-TCP1* construct was used as a negative control. (C) Bimolecular fluorescent complementation assays in onion epidermal cells by particle bombardment. TCP14 and DOF6 were fused to the C- and N-terminal fragments, respectively, of yellow fluorescent protein (YFP) and co-bombarded over epidermal onion cells. Images were obtained with a fluorescence microscope.

Discussion

DOF6 *is regulated by after-ripening and enhances* ABA1 *expression*

In this study, DOF6 has been demonstrated to negatively affect seed germination, most probably by increasing the expression of ABA-related genes and seed ABA content (Supplementary Fig. S5). This regulation is transient and conditioned by the AR status of the seed. TCP14 has been identified as an interactor of DOF6, and *tcp14* seeds displayed phenotypes similar to those observed for *ioex-DOF6*. These similarities extend to the expression of ABA-related genes and could explain a possible regulatory mechanism for their AR-dependent phenotypes and opposing roles.

DOF6 transcripts were abundant in FH dry seeds and decreased upon imbibition and during seed after-ripening. In a systematic study of *Arabidopsis* TFs, Barrero *et al.* (2010) identified 39 TF genes that were differentially expressed between FH and AR seeds after short imbibition periods, and suggested that they had different roles in seed dormancy control: genes up-regulated in AR seeds (AR set) would be related to germination and dormancy release, whereas genes up-regulated in FH seeds (FH set) would be involved in dormancy maintenance. Although other DOF genes were highly represented in the AR set, *DAG1* among them, *DOF6* was not included in any of the sets. However, our data showed that *DOF6* was consistently down-regulated by after-ripening and should be, consequently, included in the FH set.

Some DOF TFs have been shown to play dual and/or opposing roles during seed maturation and upon germination in barley: BPBF and HvDOF19 are negative regulators of hydrolase genes expressed in the aleurone of germinating seeds, but are transcriptional activators of protein storage genes during seed maturation (Mena *et al.*, 1998, 2002; Moreno-Risueño *et al.*, 2007a) and SAD and HvDOF17 are an activator and a repressor, respectively, in both phases of seed development (Isabel-LaMoneda *et al.*, 2003; Diaz *et al.*, 2005; Moreno-Risueño *et al.*, 2007a). In *Arabidopsis*, DAG1 is a DOF TF negatively regulating seed germination (Papi *et al.*, 2000; Gualberti *et al.*, 2002; Gabriele *et al.*, 2010). Despite the phylogenetic closeness between *DAG1* and *DOF6* and their similar effects on germination, they must fulfil different roles, since *DAG1* was up-regulated in AR compared to FH imbibed seeds (Barrero *et al.*, 2010). In addition, *dag1* KO seeds did not show altered sensitivity to ABA (Gualberti *et al.*, 2002).

Barrero *et al.* (2010) analysed loss-of-function mutants for 22 genes belonging to the AR and FH sets. Only two of these mutants, belonging to the AR set, displayed altered

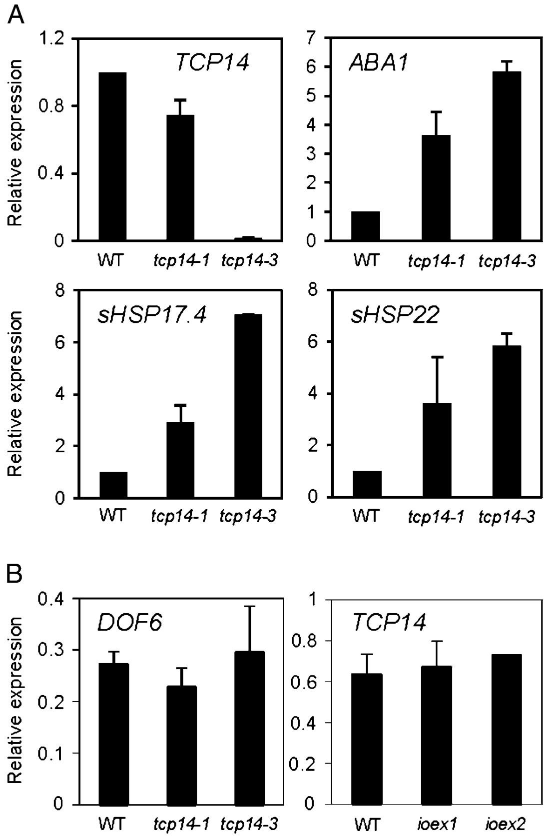

Fig. 7. mRNA levels of *DOF6* and ABA-related genes in germinating *tcp14* mutant seeds. (A) Expression of *TCP14*, *ABA1*, *sHSP17.4*, and *sHSP22* was compared by quantitative RT-PCR between freshly harvested wild-type (WT) and *tcp14* imbibed seeds. WT expression levels are 1 in all cases. (B) mRNA levels of *DOF6* and *TCP14* were quantified in *tcp14* and *ioexDOF6* FH seeds, respectively. WT and *ioexDOF6* seeds used in the left panel were imbibed for 24 h in 50 μM estradiol. Gene-specific mRNA levels are relative to *UBC21*.

dormancy, indicating a high degree of gene redundancy and a high robustness of the dormancy programme. This suggests that gain-of-function approaches, such as the one used in this study, may be more informative when analysing contributions of TFs whose loss-of-function do not show altered phenotypes, or cause lethality. The lack of insertion mutants in public databases together with the impossibility to silence *DOF6* by amiRNAs and ihpRNAs, suggested that the reduction in *DOF6* mRNA levels may be incompatible with plant viability.

The germination delay of FH *ioexDOF6* seeds was accompanied by the upregulation of the ABA biosynthetic gene *ABA1*. *ABA1* encodes a zeaxanthin epoxidase, the first enzyme in ABA biosynthesis, which has also been described to regulate ABA accumulation during seed development (Audran *et al.*, 2001). Mutants deficient on this gene have been reported to show a non-dormant phenotype (Koornneef *et al.*, 1982), whereas *ABA1* over-expression affects ABA levels and delays seed germination (Frey *et al.*, 1999; Park *et al.*, 2008). In addition, *ABA1* is down-regulated by after-ripening and it has been suggested to play a key role in dormancy maintenance in non-AR seeds (Carrera *et al.*, 2008), whereas mutants deficient in ABA biosynthesis and ABA-signalling pathways show altered dormancy and germination phenotypes (Finkelstein *et al.*, 2002; Rodriguez-Gacio *et al.*, 2009). DOF6 must also play a role in dormancy control since WT germination was restored in FH *ioexDOF6* seeds when dormancy-breaking treatments were applied upon imbibition (Supplementary Fig. S4). Other genes up-regulated in *ioexDOF6* FH imbibed seeds were sHSPs. These genes are up-regulated by ABA and are normally expressed during seed maturation and have increased expression levels in imbibed dormant seeds (Wehmeyer *et al.*, 1996; Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Kotak *et al.*, 2007; Carrera *et al.*, 2008).

DOF6 mRNA levels were down-regulated by after-ripening and this might occur through silencing with an AR-induced miRNA. Reyes and Chua (2007) found that the mRNAs encoding two positive regulators of ABA responses (MYB TFs) were cleaved by the miRNA159 during germination in response to ABA. Regarding *DOF6*, no miRNA predicted to regulate *DOF6* mRNA levels was found by *in silico* analysis (http://asrp.cgrb.oregonstate.edu/). Increased levels of *DOF6* mRNAs have been demonstrated to be sufficient to produce delayed germination and ABA-related gene expression in FH but not in AR seeds.

Biological relevance of the DOF6–TCP14 interaction

A new interaction between TF members of the DOF and TCP families has been uncovered. TCPs are plant-specific TFs that have been associated with promotion of cell growth and proliferation, hormone biosynthesis, and circadian rhythms and they have been shown to interact with armadillo BTB, NAC and TOC1 proteins (Martin-Trillo and Cubas, 2010). The biological significance of the DOF6–TCP14 and its role in after-ripening and ABA biosynthesis pathways is supported by several lines of evidence: (i) interaction of DOF6 and TCP14 was observed in the nuclei of plant cells where TFs usually exert their functions; (ii) both TFs were expressed in dry seeds and in the vascular tissues of the embryo during seed imbibition (this work; Tatematsu *et al.*, 2008); (iii) *ioexDOF6* and *tcp14* KO mutants had increased expression of specific genes involved in ABA biosynthesis and ABA-mediated stress responses; (iv) *ioexDOF6* and *tcp14* mutant FH seeds had a delayed germination phenotype which is abolished by after-ripening (this work; Tatematsu *et al.*, 2008); v) *ioexDOF6* and *tcp14* AR seeds had ABA-hypersensitive germination (this work; Tatematsu *et al.*, 2008); and (vi) *p35S:DOF6* plants showed growth retardation and a reduced plant stature as observed in the *tcp14 tcp15* double mutant (Kieffer *et al.*, 2011). These results suggest that DOF6 and TCP14 have opposite functions. Since they do not regulate their transcript levels reciprocally (Fig. 7B), and *TCP14* mRNA levels do not change during after-ripening (Supplementary Fig. S6), it could be possible that, in FH seeds, DOF6 abundance would exceed that of TCP14 (Supplementary Fig. S7) and DOF6 proteins not 'sequestered' in the interaction with TCP14 would be able to promote the expression of ABA-related genes. The balance of this TF abundance would be shifted at the end of after-ripening and would allow TCP14 to promote cell proliferation and germination. Another possibility could be the existence of other factors that may favour or oppose DOF6 function. Good candidates would be those regulated by the AR status of the seed. Out of the 39 TFs identified by Barrero *et al.* (2010), 29 are present in this study's TF library (Castrillo *et al.*, 2011). However, no interaction between DOF6 and any of these TFs was observed.

Besides its putative role in seeds, it is possible that DOF6 participates in other developmental and physiological processes, since its mRNA is present in flowers, leaves, and roots (Fig. 1), specifically in the vascular system (Supplementary Fig. S8). This is in agreement with the severe growth defects produced when *DOF6* is constitutively over-expressed (Fig. 2). It was also observed that the lack of progeny in *p35S::DOF6* plants was due to reduced pollen viability (Supplementary Fig. S2), although this could be an indirect effect. Other TFs involved in seed dormancy and germination are also involved in the regulation of development outside the seed. For instance, SPT is involved in carpel and fruit development (Groszmann *et al.*, 2008), ABI5 is involved in growth-insensitivity to ABA (Lopez-Molina and Chua, 2000), and TCP14 promotes cell division in young internodes (Kieffer *et al.*, 2011). In this report, the inducible approach has allowed the study of *DOF6* function in the context of dormancy and germination and isolated from other possible roles in the plant.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Oligonucleotide sequences and gene loci.

Supplementary Fig. S1. Strategies used to obtain *DOF6* loss-of-function lines.

Supplementary Fig. S2. Constitutive over-expression of *DOF6* produces plant sterility.

Supplementary Fig. S3. *DOF6* expression levels and germination kinetics in three independent *ioexDOF6* lines.

Supplementary Fig. S4. Dormancy-breaking treatments eliminate FH *ioexDOF6-delayed* germination.

Supplementary Fig. S5. Effect of *DOF6* over-expression on ABA levels in imbibed seeds.

Supplementary Fig. S6. *TCP14* mRNA levels in FH and AR seeds.

Supplementary Fig. S7. Proposed model of the physiological consequences triggered by the interaction between DOF6 and TCP14 during *Arabidopsis* seed germination.

Supplementary Fig. S8. Expression of the GUS reporter gene driven by the *DOF6* promoter in different plant organs.

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