Title: **Multifaceted Role of Cycling Dof Factor 3 (CDF3) in the regulation of flowering time and abiotic stress responses in Arabidopsis**

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ABSTRACT

DOF (DNA-binding with one finger)-type transcription factors are involved in many fundamental processes in higher plants, from responses to light and phytohormones to flowering time and seed maturation, but their relation with abiotic stress tolerance is largely unknown. Here, we identify the roles of CDF3, an Arabidopsis *DOF* gene in abiotic stress responses and developmental processes like flowering time. *CDF3* is highly induced by drought, extreme temperatures and abscisic acid treatment. The CDF3 T-DNA insertion mutant *cdf3-1* is much more sensitive to drought and low temperature stress, whereas *CDF*3 overexpression enhances the tolerance of transgenic plants to drought, cold and osmotic stress and promotes late flowering. Transcriptome analysis revealed that CDF3 regulates a set of genes involved in cellular osmoprotection and oxidative stress, including the stress tolerance transcription factors CBFs, DREB2A, and ZAT12, which involve both GIGANTEA-dependent and independent pathways. Consistently, metabolite profiling disclosed that the total amount of some protective metabolites including GABA, proline, glutamine and sucrose were higher in CDF3-overexpressing plants. Taken together, these results indicate that CDF3 plays a multifaceted role acting on both flowering time and abiotic stress tolerance, in part by controlling the CBF/DREB2A-CRT/DRE and ZAT10/12 modules.

Short Abstract

The present study provides new notions about the function of DOF Transcription factors and unveils CDF3 as a key factor that display multiple roles related to plant responses to adverse environmental conditions and the developmental program underlying the transition from vegetative to reproductive phase.

Key words: Drought stress, low temperature stress, nitrogen, flowering time, DOF, CDF, gene expression, *Arabidopsis.*

INTRODUCTION

Abiotic stresses such as drought and extreme temperatures are among the most important environmental factors that limit plant growth, development and productivity. Plants have developed sophisticated molecular, biochemical and physiological mechanisms to adjust growth according to the availability of resources and to environmental conditions (Xiong *et al.,* 2002; Zhu*,* 2002; Shinozaki and Yamaguchi-Shinozaki, 2007; Ahuja *et al.,* 2010; Skirycz and Inze 2010; Osakabe *et al.,* 2011; Nishiyama *et al.,* 2012). Transcriptome analyses have identified a number of genes that are inducible by abiotic stresses (Seki *et al.,* 2002; Shinozaki *et al.,* 2003; Yamaguchi-Shinozaki and Shinozaki, 2006) encoding for proteins with function in stress tolerance, including osmoregulatory and antioxidant proteins, chaperones, detoxification enzymes and LEA (Late Embryogenesis Abundant) proteins (Yamaguchi-Shinozaki and Shinozaki, 2004; 2006; Gong *et al.,* 2010) and genes involved in signal transduction and the control of gene expression, such as protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism (Seki *et al.,* 2003; Shinozaki and Yamaguchi-Shinozaki, 2006) and various transcription factors (TFs). These stress-inducible transcription factors are members of the DREB, ERF, WRKY, MYB, bHLH, bZIP, DOF and NAC families (Shinozaki *et al.,* 2003).

The DOF (DNA binding with One Finger) proteins are a group of plant-specific TFs that contain a DNA-binding domain usually located close to the N-terminal region of the protein. This DOF domain is a highly conserved region of 52 amino acid residues with a C_2-C_2 finger structure associated to a basic region that binds specifically to *cis* regulatory elements containing the common core 5´-T/AAAG-3´ motif (Yanagisawa and Schmidt, 1999; Noguero *et al.,* 2013). In contrast, the C-terminal end is a highly variable region that contains the transcriptional regulatory elements and it might

reflect diverse functions of different DOF proteins (Yanagisawa 2001; Hong-Feng *et al.,* 2013).

In previous studies, DOF proteins have been reported to be involved in the regulation of a variety of biological processes including seed maturation, germination and hormone signaling (Reviewed by Yanagisawa 2002; Noguero *et al.,* 2013). Moreover, DOF TFs such as maize DOF1 and DOF2 have also been involved in the control of carbon and nitrogen metabolism through the regulation of phosphoenolpyruvate carboxykinase (PECPK), glutamine synthase (GS) and glutamate synthase (GLU) (Yanagisawa and Sheen 1998; Yanagisawa *et al.,* 2004; Rueda-Lopez *et al.,* 2008; Kurai *et al.,* 2011).

Arabidopsis genome encodes 36 DOF TFs. Phylogenetic studies using the complete set of amino acid sequences of DOF proteins from Arabidopis, rice, tomato and *Brachypodium,* identified four major clusters of orthologous genes or subfamilies (A, B, C and D) (Lijavetzky *et al.,* 2003; Hernando-Amado *et al.,* 2012; Corrales *et al.,* 2014a). The group D contains a cluster of DOF factors whose transcripts oscillate under constant light conditions and are known as *Cycling Dof Factors* (*CDF1-5*) (Imaizumi *et al.,* 2005; Fornara *et al.,* 2009). It is well established that CDF transcription factors display an important role in the photoperiodic flowering-time control in Arabidopsis, by modulating the diurnal expression rhythm of *CONSTANS* (*CO*) expression and consequently *FT* expression. In addition, the stability of CDF proteins is compromised under long days by a protein complex including FLAVIN-BINDING KELCH REPEAT F-BOX PROTEIN (FKF1) and GIGANTEA (GI) (Imaizumi *et al.,* 2005; Sawa *et al.,* 2007; Jarillo *et al.,* 2008; Fornara *et al.,* 2009). Recently, data reported by Corrales *et al.,* (2014a) has shown that tomato CDFs (SlCDFs) display additional functions as transcriptional regulators. The overexpression of

tomato *SlCDF1* and *SlCDF3* in Arabidopsis enhanced drought and salt tolerance. Moreover, the overexpression in Arabidopsis of *SlCDF3* but not *SlCDF1* promotes late flowering by modulating the expression of *CO* and *FT*, suggesting that CDFs might play a central role in abiotic stress tolerance, along with their role in flowering time control.

In this study, we find that in Arabidopsis CDF3 is particularly induced by drought, salt, extreme temperatures and ABA. We show that *CDF3* overexpression enhances plant tolerance to drought, cold and osmotic stress, whereas down-regulation of CDF3 (*cdf3-KO*) leads to attenuated resistance. Moreover we demonstrate that CDF3 regulates a set of genes involved in cellular osmoprotection and ROS homeostasis, which are associated with changes in sugar and amino acid levels in stressed plants through both GI- dependent and -independent pathways. These findings suggest that Arabidopsis CDF3 plays multiple roles in both abiotic stress responses besides its known role in flowering time signal transduction pathways.

MATERIAL AND METHODS

Plant material and growth conditions

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as WT. The *cdf3-1* T-DNA insertion knockout mutant was obtained from the GABI-Kat collection (GK-808605; Rosso *et al.,* 2003). Seeds were surface-sterilized and stratification was performed by planting seeds on half strength MS (MS/2) medium (Murashige and Skoog, 1962) containing 0.5% (w/v) sucrose and 0.8% (w/v) agar and incubating them at 4 °C for 2 days. After germination, 10-day-old seedlings were transferred to soil and grown in a growth chamber at 22 °C under LD (16/8 h light/dark) conditions.

Plasmid constructs and Arabidopsis transformation

The open reading frame (ORF) of *CDF3* gene was cloned into a binary vector pGWB2 under the control of the 35S CaMV promoter (Karimi *et al.,* 2007). The resultant plasmid was used to transform *Arabidopsis thaliana* (Col-0) plants by the floral dip method (Clough and Bent, 1998). For β-GLUCURONIDASE (GUS) histochemical staining experiments the promoter regions of the *CDF3* and *CRUCIFERIN (CRU)* genes (from -1060bp and -1200bp to the ATG, respectively) were cloned into a binary vector containing a *GUS* reporter gene (*uidA*), producing an in-phase fusion with the reporter gene constructs *pCDF3::GUS* and *pCRU::GUS*, respectively. The corresponding plasmids were used to transform Arabidopsis plants.

Subcellular localization of CDF3 and histochemical GUS staining

For epifluorescence and light microscopy, 10-day-old Arabidopsis seedlings and onion epidermal cells were analyzed with a Confocal Laser Scanning microscope as described previously (Corrales *et al.,* 2014a). For GUS staining *pCDF3::GUS* and *pCRU::GUS* transgenic plants were used as described by Jefferson *et al,* (1987). See Appendix S1 for details.

Protoplast transformation

Mesophyll protoplasts were isolated from rosette leaves of 4-week-old-Arabidopsis plants ecotype Columbia (Col-0) grown in soil (21/18 °C, 8/16 h light/dark). Protoplast isolation and transfection was performed according to the method described previously (Alonso *et al*., 2009; Yoo *et al.,* 2007). For more details see Appendix S1.

RNA isolation, RT-PCR and qRT-PCR

Total RNA was extracted following Oñate-Sanchez and Vicente-Carbajosa, (2008). RT-PCR, and qRT-PCR analyses were carried out as described previously (Corrales *et al.,* 2014a,b; Catala *et al.,* 2011). For more detail please see Appendix S1.

Germination and post-germinative growth assay

Germination analyses and assays on post-germinative behaviour and root growth were performed as described previously (Corrales *et al.,* 2014b; Appendix S1). The assays were carried out using Col-0, *cdf3-1* and *35S::CDF3* lines. Seeds were collected at the same time and obtained from plants grown under the same conditions.

Photosynthesis and leaf fluorescence measurement

Net photosynthesis and related gas exchange variables, stomatal conductance and substomatal CO_2 were determined using an LI-6400 infrared gas analyser (LICOR Biosciences, Lincoln, USA) as described previously by (Galmes *et al*., 2007). Measurements were performed at steady state under saturating light (PAR 1000 µmol m^{-2} s^{-1}), 400 ppm CO_2, ambient temperature and a vapour pressure difference (vpd) between 1 and 2 kPa. Maximum photochemical efficiency (Fv/Fm) on dark-adapted leaves was measured using a portable pulse amplitude modulation fluorometer (MINI PAM, Walz, Effeltrich Germany). Responses to osmotic stress were performed using 3-week-old *cdf3-1*, *35S::CDF3* and Col-0 plants that were transplanted to hydroponic culture, and photosynthesis parameters were measured after 7 days of growth by adding 5% PEG-8000 (24h).

Drought and cold stress tolerance assay

Drought and cold stress assays were carried out as described previously (Corrales *et al*., 2014a; Catala *et al*., 2011). Drought stress tolerance tests were performed on plants grown in soil in individual pots. After 2 weeks, water supply was cut off for 15 days and then watering was resumed during 10 days. Plant survival rates and fresh weights were measured 10 days after the re-watering period. Freezing tolerance was analyzed by exposing non-acclimated or cold-acclimated (7 days at 4ºC) 2-week-old plants to -5 and -6ºC or -9 and-10ºC for 6 h, respectively. Tolerance was determined as the capacity of plants to resume growth 2 weeks after returning to control conditions.

Microarray analysis

Genome-wide expression studies with ATH1 array (Affymetrix) were performed using 3-week-old *35S::AtCDF3* and Col-0 plants, grown in chambers under 21/18 ºC, 16/8 h photoperiod. Three biological replicates (leaves) were harvested at zeitgeber time zero (ZT0) when lights were switched on and frozen into liquid N_2 for RNA extraction. Arrays for the different plant materials were hybridized according the Affymetrix GeneChip Expression Analysis manual (www.affymetrix.com). Differentially expressed genes in *35S::CDF3* compared to WT Arabidopsis plants were selected (1.5-fold; P value<0,05). They were functionally annotated by search in the TAIR Arabidopsis database, analysed using the e-northern expression browser tool (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_ in.cgi; Toufighi *et al*., 2005) and listed in Supplemental table S1. Venn diagrams were performed using Venny software (http://bioinfogp.cnb.csic.es/tools/venny; Oliveros, 2007). Gene Ontology analyses were performed using agriGO

(http://bioinfo.cau.edu.cn/agriGO/; Du *et al.,* 2010) and REVIGO (http://revigo.irb.hr/; Supek *et al*., 2011) software.

Pairwise comparisons were made using datasets of differentially regulated genes in *gi-100*, *cdf1235* mutants (Fornara *et a*l., 2015) and *35S::CDF3* plants and publicly available data of cold- and drought-regulated genes in Arabidopsis (Matsui *et al*., 2008). Venn diagrams were performed using Venny tools (http://bioinfogp.cnb.csic.es/tools/venny; Oliveros, 2007). For more details see Appendix S1.

Metabolomic analyses

Non-targeted and targeted metabolomics analyses were performed on 12-day-old control plants (*Col-0*) and two independent *35S::CDF3* lines. Samples were harvested at ZT0. Extraction, manipulation and mass spectrometric analysis of samples followed an adapted protocol described in Corrales *et al.,* (2014a). For more details, see Appendix S1.

RESULTS

Expression patterns of *CDF3* suggest its participation in abiotic stress responses in vegetative tissues of Arabidopsis

Previously, we have identified a group of tomato DOF TFs (SlCDFs) that exhibit specific expression patterns in response to diverse environmental stresses and display functions related to abiotic stress tolerance and flowering time (Corrales *et al*., 2014a). In order to identify *DOF* genes that could be involved in the regulation of

plant responses to different abiotic stresses in Arabidopsis, we examined expression patterns of the complete set of Arabidopsis *DOF*-encoding genes in plants exposed to drought, salinity, osmotic, extreme temperatures or oxidative stresses using transcriptomic data available from public databases. We found that *DOFs* genes of D group are differentially expressed in different vegetative tissues such as roots and leaves in response to some of the treatments (Fig. S1). Interestingly, among them the set of *Cycling Dof Factors* (*CDF1–5*) was particularly highly induced under some of those stresses. The strong and fast response of *CDF3* indicated that this TF might be a regulator of abiotic stress responses in Arabidopsis. In this study the function of CDF3 was further characterized.

To confirm that *CDF3* expression is controlled by different environmental cues, we performed detailed qRT-PCR expression analyses using RNA isolated from 3-week-old *Arabidopsis* plants that had been subjected to different abiotic stresses such as salinity, high and low temperatures, dehydration and also to exogenous ABA treatments for different periods of time. Transcript levels of *CDF3* in leaf tissues are significantly increased in response to temperature stress, dehydration, salinity, and exogenous ABA treatment although with different dynamics and extents (Fig.1ab). Higher levels of *CDF3* transcripts were observed in response to extreme temperatures, dehydration and ABA treatment, showing an earlier induction in response to dehydration and reaching maximum levels at 4h. By contrast, induction of *CDF3* was also observed in leaf tissues under salt treatment, with a retarded profile, reaching maximum levels at 24h.

In order to perform a deeper analysis of the spatial expression patterns of *CDF3* in response to abiotic stress, a 1-kb region upstream of the *CDF3* transcription start site was fused to the *uidA* coding sequence to generate the *pCDF3::GUS* reporter

that was transformed into wild-type plants (WT). A significant GUS staining was detected in vascular systems of leaves and stems, guard cells, pollen and petals (Fig. 1c). Interestingly, the *CDF3* promoter also produced a strong signal in mature seeds, showing maximum levels of expression at a later maturation stage as compared to the GUS staining pattern observed for the seed *CRUCIFERIN* gene (*pCRU::GUS*, Fig. 1e; Suzuki *et al.,* 2001). Further qRT-PCR experiments confirmed the observed *CDF3* expression profile (Fig. 1d). When 3-weeks-old *pCDF3::GUS* transgenic plants were exposed to different abiotic stresses such as low and high temperatures, dehydration and ABA or high salt treatments, *GUS* expression increased with very similar patterns in all cases, regardless of the treatment. GUS staining was detected in leaves, stems as well as main and lateral roots, being especially strong in vascular bundles (Fig. 1f). All these data indicate that the expression of *CDF3* is dynamic during plant development, and also in response to different abiotic stresses and that its regulation occurs at least partly at the transcriptional level.

CDF3 protein localizes to the cell nucleus and displays specific DNA-binding and activation properties

To investigate the subcellular localization of the CDF3 protein, different translational fusions of the ORF to the C-terminus of GFP were made. These constructs, driven by the *35S CaMV* promoter, were used for transient expression assays by particle bombardment of onion epidermal cells and for transformation of Arabidopsis plants. Figure 2a shows that the GFP-CDF3 fusion protein was mainly localized in the nuclei of onion epidermal cells. In contrast, the GFP control was observed in both nuclei

and cytoplasm of these cells. Similar results were obtained in stable transgenic plants (Fig. 2b).

To gain deeper understanding of CDF3 function, we analyzed its activation properties in transient expression assays in Arabidopsis protoplasts. To this end, effector plasmids containing Arabidopsis *CDF3* driven by the *35S* promoter (*35S::CDF3*), and the previously characterized tomato *SlCDF3* (*35S::SlCDF3*; Corrales *et al.,* 2014a) as positive control, were co-transfected with a reporter plasmid that contains the reporter *LUCIFERASE* gene (*LUC*) under control of a minimal promoter containing either the native *DOF* cis acting element (p*4XDOF::LUC*) or its mutagenized version (p*4xDOFmut::LUC*). The results shown in Figure 2C, indicated that AtCDF3 and SlCDF3 promoted *LUC* reporter gene expression of the construct harboring the native DOF cis acting element 5´-AAAG-3`, whereas they could not activates the expression of LUC when using the construct that contains the mutagenized DOF motif 5´-AGAC-3`. The data confirmed that CDF3 could bind specifically to the 5′-AAAG-3′ *cis*-DNA element, and also that moderately activates the *LUC* reporter gene as the tomato homologous gene *SlCDF3* (Fig. 2c; Corrales *et al.,* 2014a).

Overexpression of *CDF3* enhances drought and low temperature tolerance in Arabidopsis

The presented *in silico* expression analyses suggested that CDF3 might play an important role in plant responses to different abiotic stresses. To further explore this possibility, a phenotypic characterization of CDF3 gain and loss of function plants was performed by analyzing their responses under drought and osmotic stress

conditions. Arabidopsis plants overexpressing the full length *CDF3* under control of the *35S* promoter were generated, and two homozygous lines with relatively high expression levels of *CDF3* were selected for further analyses (Fig. 3a). In addition, we identified a T-DNA insertion mutant *cdf3-1* (GK808G05) with the insertion site located at position 792 from the ATG, in the middle of the DOF DNA binding domain according to the genome sequence and disruption verified by the absence of *CDF3* expression (Fig. S2). When grown in soil under standard greenhouse conditions, *cdf3-1* plants did not show apparent developmental differences relative to WT control plants (Col-0). Nevertheless, *CDF3* overexpressing plants flowered slightly later than WT control plants under LD conditions (Fig. S3). Interestingly, similar results were previously reported by Fornara *et al.,* (2009) when *CDF3* is overexpressed in companion cells using the *SUCROSE TRANSPORTER 2 (SUC2)* promoter. In addition, when plants were subjected to water deprivation for 15 days and allowed to recover for 10 days during which they were watered, WT and *cdf3-1* plants exhibited similar severe symptoms of water loss and significant wilting. In contrast, *35S::CDF3* transgenic plants were less affected, keeping healthy greener leaves. In fact, after a 10 days recovery period, *35S::CDF3* transgenic plants exhibited better survival rates and higher fresh weight than WT and *cdf3-1* plants (Fig. 3bc). These plants were also evaluated for osmotic stress tolerance in different germination and root elongation assays. We followed germination and appearance of green cotyledons in *35S::CDF3*, *cdf3-1* and WT seeds germinated on 1/2MS (control) or 1/2MS supplemented with 200 or 250mM mannitol by giving them scores after 3 and 5 days, respectively. When sown on control MS medium, all genotypes displayed similar germination behavior, but in the presence of 200 or 250mM mannitol, the germination rates were clearly higher and cotyledons greener in *35S::CDF3*

compared to WT plants, and they scored significantly lower in *cdf3-1* plants (Fig. 3d). In a second experiment, primary root elongation assays were conducted for *35S::CDF3, cdf3-1*, and WT plants grown either on 1/2MS medium (control) or 1/2MS medium supplemented with 200mM mannitol for 10 days (Fig. 3e). To evaluate growth differences between plants under control and osmotic stress conditions, data were represented as the percentage of root growth relative to standard conditions. Under control conditions, there was no difference between gain- and loss- of function lines and WT plants. In contrast, when grown on osmotic stress medium, *35S::CDF3* lines showed moderate but statistically significant higher values of relative root growth than WT plants, whereas *cdf3-1* plants exhibited lower values of relative root growth (Fig. 3e-f).

Since low temperatures rapidly induce the expression of *CDF3,* we decided to investigate whether this protein could have a role in tolerance to freezing temperatures. With this purpose, the freezing tolerance of *CDF3* gain- and loss-of-function plants was analyzed before and after cold acclimation for 7 d at 4°C. Freezing tolerance was determined in non-acclimated and cold-acclimated plants as their capacity to resume growth after being exposed for 6 h to different freezing temperatures when returned to control conditions. Interestingly, when compared to non-acclimated WT plants *CDF3* overexpressing plants show higher levels of freezing tolerance, whereas, *cdf3-1* mutants display significant lower tolerance to freezing (Fig. 4a). Moreover, after cold acclimation *35S::CDF3* lines are also notably more freezing tolerant than WT plants (Fig. 4b), while *cdf3-1* plants are impaired in their capacity to tolerate freezing. The freezing tolerance phenotypes of non-acclimated and cold-acclimated WT, *cdf3-1* and *35S::CDF3* plants are displayed in Fig. 4cd, respectively, as a representative example. These data indicate that CDF3

acts as a positive regulator of constitutive freezing tolerance and cold acclimation response in Arabidopsis.

Enhanced photosynthetic capacity of *CDF3* overexpressing plants under osmotic stress conditions.

To investigate the underlying mechanisms involved in the response of *35S::CDF3* and *cdf3-1* plants to dehydration we examined different physiological parameters such as net photosynthesis and related gas exchange variables, stomatal conductance and sub stomatal CO_2 concentration using an LI-6400 infrared gas analyzer (LICOR). Three-week-old *35S::CDF3* (L2.1), *cdf3-1* and WT plants were transplanted to hydroponic culture to facilitate osmotic stress treatment with 5% PEG-8000 for 24 hours, after which photosynthesis parameters were measured and represented as percentage to untreated control conditions. Interestingly, plants grown under osmotic stress conditions displayed genotype-dependent changes in photosynthetic rates that were not observed under control conditions. As shown in Fig. 3g, photosynthetic rates are higher in *CDF3* overexpressing plants and lower in *cdf3-1*, respectively. A similar response was observed for the stomatal conductance (g_s) (Fig. 3g). Furthermore, the higher increase in the substomatal CO_2 concentration in the control plants (292 to 315 µmol/mol) and *cdf3-1* (297 to 316 µmol/mol) compared to *35S::CDF3* plants (294 to 300 µmol/mol) under osmotic stress suggests higher biochemical limitations to photosynthesis in the lines with normal or compromised levels of *CDF3*. Accordingly, we observed a reduction in the maximum quantum yield of PSII (F_v/F_m) in *cdf3-1* and WT plants, which indicates the existence of photo-inhibition events, whereas this parameter was not affected by osmotic treatment in *35S::CDF3* plants (Fig. S4). The higher photosynthetic performance of

the *CDF3* overexpressing plants under osmotic stress conditions supports the higher growth observed in these plants under water stress.

Since stomatal conductance is greatly affected by ABA, we decided to investigate the possible role of ABA in the different responses of the stomatal conductance observed in the analyzed lines. Thus, 4-week-old *35S::CDF3* (L2.1), *cdf3-1*, and control (Col-0) plants grown in soil were analyzed by spraying with 0.5 µM ABA solution in the underside of the leaves and photosynthesis parameters were measured 1, 2 and 3.5 h after treatment. The results obtained revealed that these lines showed significant differences in photosynthetic parameters with different dynamics and extents (Fig. 3h). While control and *cdf3-1* plants exhibited a similar significant reduction of stomatal conductance values after 1 hour of ABA treatment (60% of the non-treated), *35S::CDF3* overexpressing plants exhibited a delayed response with almost no effect after 1 hour of the treatment. However at longer times (2-3h), *35S::CDF3* plants finally reach stomatal conductance values similar to WT and *cdf3-1* plants. Accordingly, the photosynthetic rate followed a similar response, showing an earlier decrease (0-2 hours) in control and *loss-of-function* plants, and delayed in *35S::CDF3* plants, although equaled to the former ones after 3.5 hours. Taken together, our data suggest that lower biochemical and stomatal limitations to photosynthesis results from high levels of *CDF3* in overexpressing plants and this may lead to bigger size under abiotic stress conditions.

Transcriptome analysis of transgenic Arabidopsis overexpressing *CDF3*

To further gain insight into the molecular mechanisms underlying the higher tolerance to drought and low temperatures associated to the *CDF3* overexpression, transcriptome analyses of three-week-old *35S::CDF3* (L2,1) and Col-0 plants were

performed using the Affymetrix Arabidopsis oligo microarray and analyzed at ZT0. The results of the study reveal that among ~24,000 *Arabidopsis* genes 531 were differentially expressed (>1.5-fold change; P value≤ 0,05) in *CDF3* overexpressing plants compared with WT plants in control conditions (Fig. 5; Table S1). About, two-thirds of the genes (409) were up-regulated, whereas 122 were down-regulated. Moreover, Gene Ontology annotation analyses of the misregulated genes in *35S::CDF3* plants revealed that the putative targets of CDF3 are highly enriched in stress-related and signal transduction categories, like “response to water deprivation”, “light intensity”, “cold”, “oxidative stress” and metabolism, like “amino acid” and “carbohydrate biosynthesis” (Fig. 5c,d), thus indicating a role of CDF3 in early stress responses. Among the upregulated genes a group of *LEA* (*Late Embryogenesis Abundant protein*), *HSP* (*Heat Shock Protein*) and *DNAJ* genes (Table S1) that function in osmotic stress regulation, protein folding and assembly processes, autophagy and protection of cellular structural integrity under extreme temperatures, osmotic and dehydration conditions (Ingram and Bartels 1996; Chen *et al.,* 2010; Sato & Yokoya, 2008; Yang *et al*., 2015) were misregulated in *35S::CDF3* plants, indicating the participation of CDF3 in osmoprotection. Using the e-northern Expression Browser tool (Toufighi *et al.,* 2005), we performed a detailed classification of the identified genes that revealed that many of them are regulated by different abiotic stresses (Fig. 5a). In fact, among the up-regulated genes 337, 109, 147 and 76 were significantly misregulated (>1.5-fold) in at least one time-point during drought, low temperature, salinity and osmotic stresses, respectively (Fig. 5a). Remarkably among them is included a group of genes previously reported to be involved in cold and drought stress responses, such as *cold-regulated-genes COR78/RD29A, COR15a, COR413, KIN1 and EARLY RESPOSIVE TO*

DEHYDRATION (ERD-7,-10,-12 and -15), GALACTINOL SYNTHASE (GOLS2) and the *SUCROSE SYNTHASE 1 (SUS1)* gene (Déjardin *et al*., 1999; Kim and Nam, 2010; Kiyosue *et al*., 1994; Thomashow, 2010; Taji *et al*., 2002; Table S1). Thus, these data suggest that CDF3 might function in the regulation of cellular integrity, metabolism and oxidative ROS homeostasis to control cellular and oxidative damage promoted by drought and low temperatures.

A detailed exploration of the previously described CDF3 regulon of 531 target genes allowed the identification of important genes encoding key regulatory transcription factors reported to participate in different abiotic stress responses: DREB2A (Sakuma *et al.,* 2006), WRKY46 (Ding *et al.,* 2014), ERF6 (Dubois *et al.,* 2013) and WRKY30 (Scarpezi *et al.,* 2013) involved in drought and osmotic stress signaling, CBF1, CBF2, CBF3 (Liu *et al.,* 1998; Medina *et al*.,1999; Novillo *et al.,* 2007) and ZAT6 (Shi *et al.,* 2014) involved in low temperature stress, and ZAT10 and ZAT12 (Davletova *et al.,* 2005; Mittler *et al.,* 2006) involved in oxidative stress. These results indicate that CDF3 might play an important role organizing abiotic stress responses by controlling the expression of key stress-related transcription factors. Quantitative RT-PCR was performed to confirm some of the identified differentially expressed genes in the *35S::CDF3* plants. In this analysis we included both classical abiotic stress-responsive genes such as *COR15A*, *RD29A* and *ERD10* and the transcriptional regulators *CBF1, CBF2, CBF3, ZAT10, ZAT12* and *DREB2A*. Figure 6ab shows the expression levels of the analyzed genes in *35S::CDF3* transgenic lines, where they exhibit higher values (from two- to four-fold) than in the WT plants. These data confirmed the results of the chip experiments and indicate that *CDF3* might be an upstream activator in drought and low temperature stress pathways,

acting directly or indirectly on the expression of different stress-regulated target genes.

It has been reported that mutations in *GI* increased CDF abundance preventing the expression of CO and FT, promoting late flowering and the increase tolerance to oxidative stress (Fornara *et al*., 2009, 2015). By contrast, the multiple mutant *gi-100-cdf1235* suppresses late flowering, oxidative stress tolerance of *gi* and restores expression patterns of *CO* and *FT*. To study the overlap of the GI-CDF module and determine the specific contribution of CDF3, we compared the datasets of the differentially expressed genes in *gi* and *cdf1235* mutants obtained from non-stressed plants (Fornara *et al*., 2015) with the ones from *35S::CDF3* plants. In the case of the *gi* mutant, whose CDFs appear upregulated, we found that among the misregulated genes about 12% were also differentially expressed in *35S::CDF3* plants (Table S1-2, Fig. S5). Notably, we observed that a large set of the genes that are common between *35S::CDF3* and *gi* transcriptional profiles, about 83.4% (Table S3), are upregulated, which could be expected considering that both lines present high levels of expression of *CDF3*. A similar comparison with the *cdf1235* multiple mutant, shows that a limited number of genes is upregulated in the *CDF3* overexpessor and repressed in *cdf1235* plants, suggesting that CDF3 might regulate this specific set of genes (Table S3).

To analyze the contribution of the GI-CDF module to drought and low temperature responses and precisely determine the genes that depend on GI and CDF3, we compared datasets of differentially regulated genes in the *gi* mutant and *35S::CDF3* plants, with publicly available data obtained from Arabidopsis plants exposed to drought and low temperature stress treatments (Matsui et al 2008). Notably, a limited overlap between stress-responsive- genes regulated by GI and CDF3 was observed

(Fig S6). Actually, in the case of *35S::CDF3* plants, 92.6% and 89.7% of the drought and low temperature regulated genes identified, respectively, are specific for CDF3, and were not differentially expressed in *gi* mutant (Fig S6). Similarly, in the *gi* mutant, about 83% and 84% of the drought and low temperature regulated genes identified are not misregulated in *CDF3* overexpressor plants (Fig. S6). Altogether, these data suggest that a significant set of genes regulated by the GI-CDF module are under direct control of CDF3, but also that CDF3 specifically modulates the expression of certain genes in a GI-independent fashion.

To elucidate whether *CDF3* might directly regulate abiotic stress responsive genes, we first searched for common *cis*-acting elements present in the promoters of the *CDF3* misregulated genes using the Promomer tool (Toufighi *et al.,* 2005) and found overrepresentation of the DOF DNA-binding motif 5´-T/AAAAG-3´ in their promoter regions (Fig. S7). Among these genes the *COR15* promoter was selected for further studies as a potential target of CDF3. Using protoplast transformation, a *35S::CDF3* effector plasmid was cotransfected with a reporter plasmid harboring *the uidA* reporter gene under control of 1kb promoter region of *COR15* containing multiple DOF *cis*-DNA binding elements (Fig. S8). As shown in Fig. 6c, CDF3 activates the expression of the reporter gene, most likely through one of the DOF binding sites present in the *COR15* promoter. To confirm the potential role of the *DOF* binding site as an abiotic stress response cis-acting element component, the *uidA* gene under control of a minimal promoter containing a 2×DOF *cis*-DNA element (*pBT10 2×DOF-GUS*) was used to transform *Arabidopsis* protoplasts incubated under different stress conditions such as extreme temperatures (4ºC and 37ºC) or treated with NaCl (25mM) and ABA (100µM) for 12h (Fig. 6d). Notably, higher levels of GUS activity compared to the untreated control were observed under low and high temperature

treatments (>1,5-fold), slightly higher levels were detected under salt treatment and no differences were found in ABA treatments. These results obtained indicate that the 5′-AAAG-3′ DOF binding site might function as a *cis*-acting abiotic stress response element and supports the role of the CDFs as candidate nuclear trans-acting factors operating on it.

The overexpression of *CDF3* in vegetative tissues impacts sugar and amino acid metabolism

Drought and extreme temperatures are conditions that promote substantial changes in plant physiology and metabolism (Rizhsky *et al.,* 2004; Seki *et al.,* 2007; Chaves *et al.,* 2009). To investigate whether *CDF3* overexpression in Arabidopsis promotes changes in the plant metabolome that would be consistent with the higher drought tolerance, we performed metabolomic analyses of these plants. In a first step, a non-targeted metabolite analysis of *35S::CDF3* (lines L2.1 and L5.4) and WT plants was carried out. The different samples were compared by principal component analysis (PCA) considering about 1000 molecular features per sample. The results revealed that both *35S::CDF3* overexpressor lines exhibited a significant alteration of the metabolome (Fig. 7a). To further dissect these changes we performed a targeted metabolomic profiling by gas chromatography-mass spectrometry (GC-MS) to study the relative levels of different polar compounds, including proteinogenic amino acids as well as other amino acids and distinct sugars, extracted from 12-day-old WT and *35S::CDF3* plants grown under non-stress conditions. As shown in Fig. 7c and Table S4, comparison of the GC-MS profiles revealed a number of clear differences between WT and overexpressing lines. Overexpression of *CDF3* in Arabidopsis

significantly induced the accumulation of sugars like sucrose (1.1-fold) and glucose (2-fold) and amino acids like L-leucine (1.3-fold), L-asparagine (1.82-fold), L-glutamine (1.53-fold), γ-aminobutyric acid (GABA; 1.3-fold) and L-proline (2.2-fold), previously associated to stress tolerance and indicative of increased nitrogen assimilation, as reported for other *DOF* genes such as *ZmDOF1* (Yanagisawa *et al*., 2004).

DISCUSSION

During the last decade different reports have implicated the DOF transcription factors in the regulation of biological processes related to plant growth and development. In this work we have identified a group of *Arabidopsis DOF* genes known as CDFs whose expression responds to different abiotic stresses like salt, drought, and extreme temperatures. The results of our study provide functional evidence in support that one of them, CDF3, contributes to processes such drought, osmotic and cold stress tolerance and flowering. CDF3 displays spatially separated functions modulating the expression of different sets of genes that operate in both GIGANTEA-dependent and -independent pathways.

Novel disclosed functions of CDF3 in abiotic stress responses

In this work we analyzed the expression patterns of the complete set of 36 genes encoding DOF proteins of Arabidopsis (Lijavetzky *et al.,* 2003), and found that those included in the group D, are highly expressed in response to different abiotic stress conditions. Interestingly, among them, the *CDFs* seemed to be regulated by drought, salinity and extreme temperatures. However, under stress conditions they display dissimilar patterns in timing of response and in spatial expression in roots and

shoots, suggesting a distinct participation in specific responses to environmental changes.

To further explore this aspect, we have performed a detailed expression analysis of *CDF3* using promoter-GUS fusions and qRT-PCR, and observe that it is rapidly induced in leaves and roots in response to different abiotic stresses, with similar expression patterns regardless of the treatment. This behavior indicates that CDF3 may respond to an intermediate common effector shared by the different stress treatments. Moreover, the observation that under abiotic stress conditions *CDF3* extends its expression pattern to leaves and roots from the vascular tissues, implies that it might play additional roles under non-stress conditions, as reported for its involvement in the control of flowering. In addition, it might suggest that CDF3 functions are spatially separated, by which flowering time is controlled in the companion cells of the phloem, whereas stress responses might take place in alternative tissues. Consistently, the flowering regulators, CO and FT, two direct targets of CDFs are controlled precisely in the vasculature of leaves (Fornara *et al*., 2009), suggesting that CDFs might display additional functions related to abiotic stress responses through alternative targets or the interaction with different factors in other tissues.

To clarify the specific participation of CDF3 in response to abiotic stress conditions we performed a functional characterization of Arabidopsis gain- and loss-of-function mutants. Phenotypic analyses, including survival rates and root length assays under stress conditions, showed that the *CDF3* T-DNA insertion mutant displays reduced tolerance to drought and low temperatures. In contrast, *CDF3* overexpressing lines are more tolerant to drought, osmotic and low temperatures, indicating that CDF3 plays multiple roles to confer protection from different abiotic stresses. This finding is

also supported by recent work reporting that a multiple *cdf1235* mutant exhibits higher sensitivity to oxidative stress promoted by methylviologen (Fornara *et al*., 2015). However, the authors observed increased susceptibility to low temperature stress in the *cdf1235* mutant mainly in the *gi* background, suggesting a complex interaction between GI and the CDFs, and that GI might need the participation of the CDFs, or a specific CDF member, to play some of its roles in response to low temperatures. The work presented here extends Fornara's observations and provides functional evidence that CDF3 plays a key role in plant responses to abiotic stresses such as low temperature, osmotic and drought, both through GI-dependent and -independent pathways.

The transcriptional profiling performed in this study revealed that about half of the differentially expressed genes in *35S::CDF3* are related to responses to osmotic, drought or extreme temperatures, which is in agreement with the phenotypes displayed by the *CDF3* overexpressing plants under abiotic stress conditions. Interestingly, *CDF3* overexpression upregulates a group of genes encoding LEAs, HSPs and DNAJ proteins that have been involved in osmotic regulation, protein folding, autophagy and protection of cellular structures under abiotic stresses (Ingram and Bartels, 1996; Wang *et al.,* 2004; Chen *et al*., 2010), in support of a role of CDF3 in the regulation of protective mechanisms against cellular damage caused by osmotic and low temperature stress. These results are in agreement with previous data of the *gi* mutant showing increased stability and accumulation of CDF proteins, expression of *COR* stress-regulated genes and increased protection to low temperatures (Fornara *et al*., 2015). However, the transcriptomic analyses reported here showed a limited overlap between stress-responsive-genes regulated by GI and CDF3. It is worth mentioning that sampling of plant materials of the *35S::CDF3* and

gi mutants for transcriptomic analyses (this work and Fornara *et al.,* 2015) was done at ZT0 and ZT12 respectively, when native *CDF3* and *GI* mRNA expression levels in the corresponding controls are most contrasting. Although this fact may reduce a partial overlap of commonly regulated genes, the presented results support that GI and CDF3 are involved in multiple abiotic stress responses, and display specific functions in drought and low temperatures stresses, most likely by controlling the expression of different sets of genes involved in organ-specific stress responses.

Metabolic profiling of *CDF3* overexpressing plants revealed an increase of amino acids like proline and GABA, and sugars like sucrose and glucose, usually accumulated at higher levels in plant tissues exposed to extreme temperatures, osmotic stress or drought (Rizhsky *et al.,* 2004; Gill and Tuteja, 2010; Hussain *et al.,* 2011). These metabolites function in osmotic adjustment, protection of membranes and ROS scavenging (Rajasekaran *et al.,* 2000; Claussen, 2005; Munns and Tester, 2008; Farrant and Moore, 2011) and their increased levels are in agreement with the higher tolerance to abiotic stress displayed by the *CDF3* overexpressing plants. This finding is also supported by recent work of Fornara *et al.* (2015) reporting that a multiple *cdf1235* mutant exhibits higher sensitivity to oxidative stress and reduced expression of several cold-regulated genes. Overall, our results demonstrate the participation of CDF3 in plant responses to different abiotic stress conditions, and also that individual CDFs might regulate specific target genes in response to particular environmental perturbations.

Impact of CDF3 on carbon and nitrogen metabolism

The expression analysis of *CDF3* revealed a complex pattern, being detected in different tissues during development. Particularly, it is highly expressed in organs and tissues with different sink/source activities such as shoots and roots, and reproductive structures like flowers and seeds. This may indicate that CDF3 plays tissue-specific functions by controlling the expression of genes involved in particular metabolic processes. In this respect, metabolite analyses of *35S::CDF3* plants revealed that under control conditions the transgenic lines exhibit important changes, including higher levels of sugars such as sucrose and glucose, and the accumulation of different amino acids such as glutamine, asparagine, proline and GABA. These observations are in agreement with previously reported metabolomic analyses of *sex3* mutant (*gi* allele) which showed higher levels of several aminoacids, sugars and sugar alcohols relative to wild type (Messerly *et al*., 2007). Interestingly, the levels of GABA, asparagine and glutamine are reliable indicators of nitrogen use efficiency (Stitt and Krapp, 1999; Yanagisawa *et al.,* 2004; Foyer *et al.,* 2006). In fact, GABA has been involved in nitrogen storage through the pathway that converts glutamate to succinate (GABA shunt), with a great impact in nitrogen economy of plants (Shelp *et al.,* 1999). The observed higher amino acid content in the overexpression lines might be related to an improvement of nitrogen assimilation as previously described for other DOF TFs (Yanagisawa, 2004).

On the other hand, GABA and the GABA shunt in plants have been connected with other functions related to abiotic stress, including osmoregulation (Shelp *et al.,* 1999), cytosolic pH regulation (Snedden *et al.,* 1995), protection against oxidative stress (Bouche *et al.,* 2003) and maintenance of the C/N balance (Shelp *et al.,* 2012; Studart-Guimaraes *et al.,* 2007). Moreover it has been shown that GABA may also

act as a putative long-distance signal molecule in the regulation of nitrate uptake (Beuve *et al.,* 2004). The metabolite profile data presented here suggests that CDF3 could participate in the regulation of the C/N metabolism favoring plant growth and development under specific stress conditions.

CDF3, a connection between flowering time and abiotic stress responses

The data presented in this work confirm the previously reported participation of CDFs in the control of flowering time. Precisely, the overexpression of *AtCDF*s in phloem companion cells (Imaizumi *et al.,* 2005; Fornara *et al.,* 2009) or the constitutive overexpression of tomato *SlCDFs* in *Arabidopsis* (Corrales *et al.,* 2014a) promote a delay in flowering time under LD conditions. Likewise, here we show that the constitutive *CDF3* overexpression not only has an impact in flowering time but also in plant responses to different abiotic stresses.

The timing of flowering, alongside with the adaptability to changing environmental conditions, has significant consequences for the reproductive success in plants. Accordingly, plants must closely integrate changes in the environment to determine the onset of flowering and ensure reproductive success. Triggering the transition from vegetative to reproductive phase relies on an extremely intricate network, linking multiple signaling pathways and regulatory proteins (Blümel *et a*l., 2015). Among them, the GI protein plays a central role in diverse signaling pathways, including circadian clock regulation photoperiodic, sugar and light signaling and stress responses (Fowler *et al.,* 1999; Gould *et al.,* 2006; Mizoguchi *et al.,* 2005; Park *et al.,* 1999; Cao *et al.,* 2005). GI activates the expression of the central flowering regulators *CO* and *FT*, by promoting the degradation of the CDFs

(Imaizumi *et al.*, 2005; Sawa *et al.,* 2007; Fornara *et al.,* 2009). Interestingly, the *gi* mutant presents higher stability and accumulation of CDF proteins, and shows increased expression of cold regulated genes and higher tolerance to cold and oxidative stress (Han *et al.,* 2013, Kurepa *et al.,* 1998; Cao *et al.,* 2005; Fornara *et al.,* 2015).

In this context, our results disclose that CDF3 controls the expression of a group of genes involved in plant responses to extreme temperatures, drought and osmotic stress, including several central abiotic stress regulators like CBFs, DREB2A and ZAT12. Interestingly, the overexpression of these *TFs* not only promotes changes in the response to different abiotic stresses but also results in late flowering (Gilmour *et al*., 2004; Vogel *et al*., 2005; Sakuma *et al*., 2006; Achard *et al*., 2005). It has been established previously that CDF3 participates in the control of flowering time through the transcriptional regulation of key factors like CO (Imaizumi *et al*., 2005; Sawa *et al*., 2007; Fornara *et al*., 2009). However, our results highlight its relation to drought and cold response pathways, and the regulatory action on CBF/DREB2A-CRT/DRE and ZAT12 modules with an impact on flowering time as well.

Finally, our metabolomic analyses reveal that *CDF3* overexpresssion promotes important changes in the plant metabolome, altering the levels of specific compounds with protective functions that alleviate detrimental effects of abiotic stress conditions. These results also would allow us to hypothesize that CDF3 might regulate the partition of C/N rich compounds depending on age, stage of plant development and environmental cues, and eventually influence the control of flowering time.

In conclusion, the present study provides new notions about the function of DOF TFs and unveils CDF3 as a key factor that display multiple roles related to plant responses to adverse environmental conditions and the developmental program underlying the transition from vegetative to reproductive phase.

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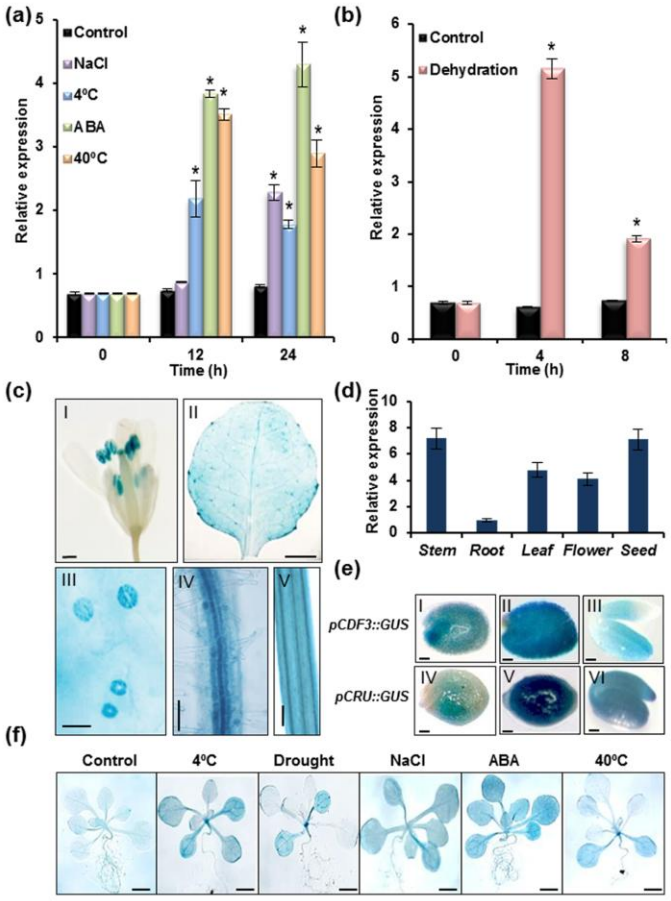

Figure 1. Expression pattern of *CDF3* gene in response to different abiotic stress conditions.

Figure. 1. Expression pattern of *CDF3* gene in response to different abiotic stress conditions.

(a-b) qRT-PCR analysis of *CDF3* gene expression. Total RNA was isolated from leaves of 3-week-old Arabidopsis plants grown under control conditions (control), treated with (A) 150mM NaCl (NaCl), low temperatures (4ºC), 100µM ABA (ABA), heat (40ºC) or (B) dried on the bench (dehydration) for the indicated periods of time. *Arabidopsis UBIQUITIN21* gene was used as a reference gene. Data are means ± SE (n=3). Asterisks indicate significant differences from control; * *P*<0.05; by Student´s t-test.

(c) GUS staining of *pCDF3::GUS* plants showing CDF3 localization in flower and pollen I, young leaf II, stomata III, root IV and stem V. Scale bars: (I) 200 µm; (II) 1mm; (III) 80 µm; (IV-V) 1 cm.

(d) Expression pattern of *CDF3* gene in different organs of adult Arabidopsis plants. qRT-PCR analyses were performed with total RNA extracted from stems, roots, leafs, flowers and seeds of 8-week-old Arabidopsis plants.

(e) GUS staining *pCDF3::GUS* (I-III) and *pCRU::GUS* (IV-VI) showing *CDF3* and *CRUCIFERIN* expression in seeds. (I, IV) early maturation, (II, V) late maturation, (III, VI) dry seeds. Scale bars: 200 µm.

(f) GUS staining showing CDF3 localization in three-week-old transgenic *pCDF3::GUS* Arabidopsis plants grown under control conditions (control) or exposed to low (4°C) or high temperature (40ºC), dried on the bench (drought), treated with 100µM ABA (ABA) or 150mM NaCl (NaCl) for 24h. Scale bars: 3 mm.

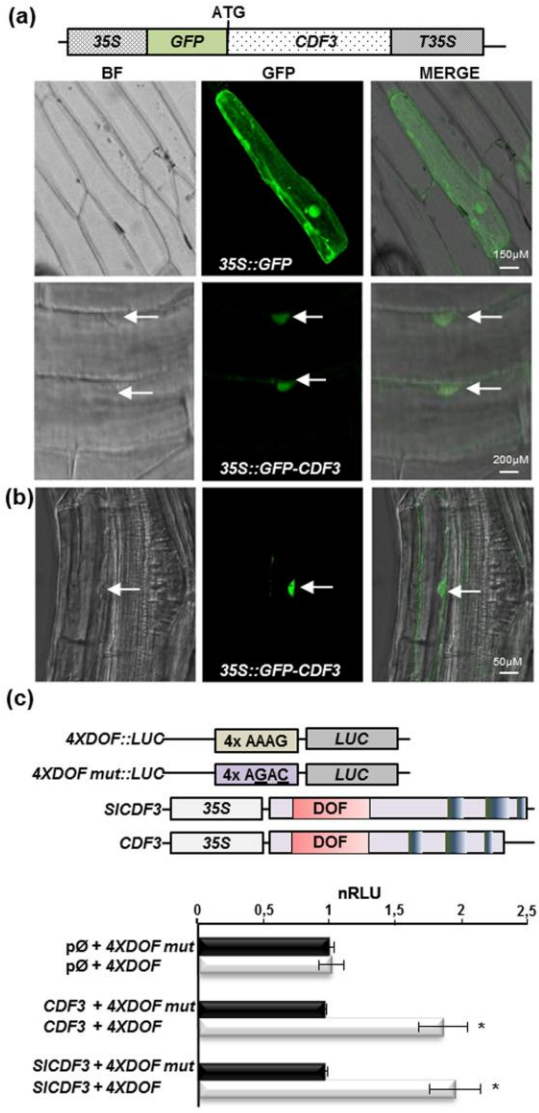

Figure 2. Subcellular localization and transcriptional activation properties of CDF3 protein.

Figure 2. Subcellular localization and transcriptional activation properties of CDF3.

(a-b) CDF3 protein is targeted to the nuclei. Onion epidermis cells (a) and Arabidopsis cells (b) were transiently and stable transformed by particle bombardment and *Agrobacterium* with *35S::GFP-CDF3* construct, respectively. As controls, onion layers were transformed with the *35S::GFP*. Confocal images of onion and Arabidopsis root cells showing CDF3 nuclear localization (White Arrows), with an overlay of the bright field (BF) and GFP images (GFP).

(c) CDF3 binding to the DOF motif. Transient expression assays in Arabidopsis protoplasts using *35S::CDF3* effector plasmid and the reporter plasmids *4xDOF::LUC* and *4XDOFmut::LUC*. Tomato homologous gene *SlCDF3* and empty effector plasmid (pϕ) were used as positive and negative controls, respectively. Data are means ± SE (n=3). Data are means ± SE (n=3). Asterisks indicate significant differences from control (pϕ); * P<0.05; by Student´s t-test.

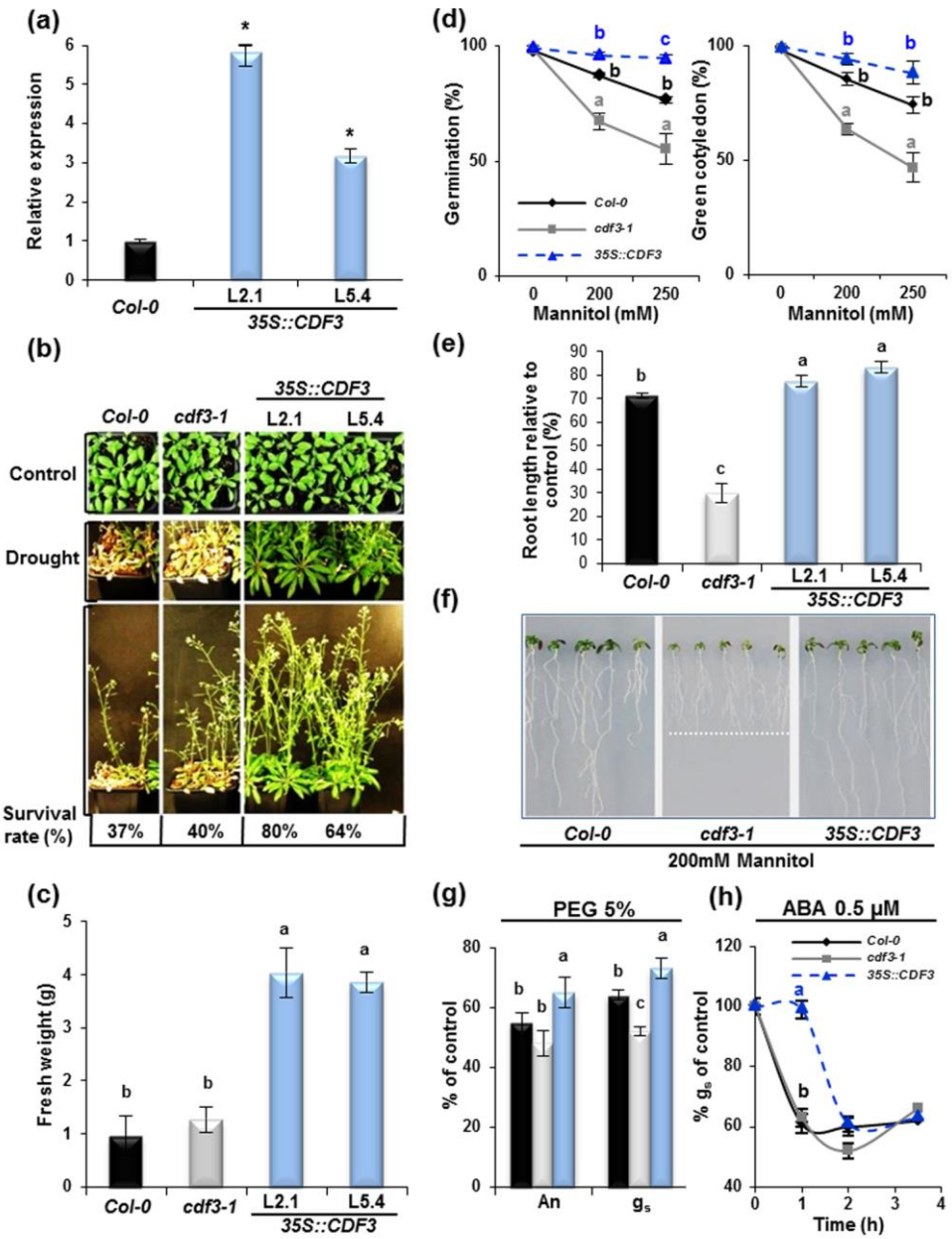

Figure 3. Effects of CDF3 on tolerance to drought and osmotic stress.

Figure 3. Effects of CDF3 on tolerance to drought and osmotic stress.

(a) qRT-PCR analysis of *CDF3* expression in *35S::CDF3* (L2.1 and L5.4) transgenic lines. Data are means ± SE (n=3). Asterisks indicate significant differences from Col-0; * P<0.05 by Student´s t-test.

(b) Phenotypes and survival rates of Col-0, mutant and overexpressor plants grown under normal and dehydration conditions. The photographs and survival rates were obtained after re-watering for 10 days after dehydration treatment.

(c) Fresh weight of Col-0, *cdf3-1* and *35S::CDF3* plants after dehydration treatment. Values are means ± SE (n=3). Letters indicate significant differences between Col-0, mutant and overexpresor plants; P<0.05; ANOVA Student-Newman-Keuls tests.

(d) Germination rates and appearance of green cotyledons of *35S::CDF3,* Col-0 and *cdf3-1* plants that were germinated under different concentrations of mannitol. Data are means ± SE (n=3). Letters indicate significant differences between Col-0, *cdf3-1* and *35S::CDF3*; P<0.05; ANOVA Student-Newman-Keuls tests.

(e-f) Root elongation assays. Six-day-old seedlings were transferred MS agar plates or supplemented with 200mM mannitol and incubated vertically for 10d before primary root length were estimated. (e) Results are represented as percentage of reduction relative to standard conditions. Data are means ± SE of three independent experiments with at least 20 plants each. Letters indicate significant differences between Col-0, *cdf3-1* and *35S::CDF3* (P<0.05; ANOVA Student-Newman-Keuls tests). (f) Representative images of Col-0, *cdf3-1* and *35S::CDF3* plants after treatments.

(g) Photosynthetic rate (An) and stomatal conductance (g_s) were estimated in 3-week-old Col-0, *cdf3-1* and *35S::CDF3* Arabidopsis plants growth under control conditions, or treated with 5% PEG, for 24h. Data were referred to the values in control conditions. Data are means ± SE (n=8). Letters indicate significant differences between Col-0, *cdf3-1* and *35S::CDF3*; P<0.05; ANOVA Student-Newman-Keuls tests.

(h) The effect of ABA on the reduction of stomatal conductance (gs) was estimated in four-week-old plants grown in soil by spraying with 0.5 µM ABA solution in the underside of the leaves and measurements were made after 1, 2 and 3.5 hours after treatment. Date are referred to the parameter at t=0. Data are means ± SE (n=6). Asterisks indicate significant differences from Col-0; * P<0.05, by Student´s t-test.

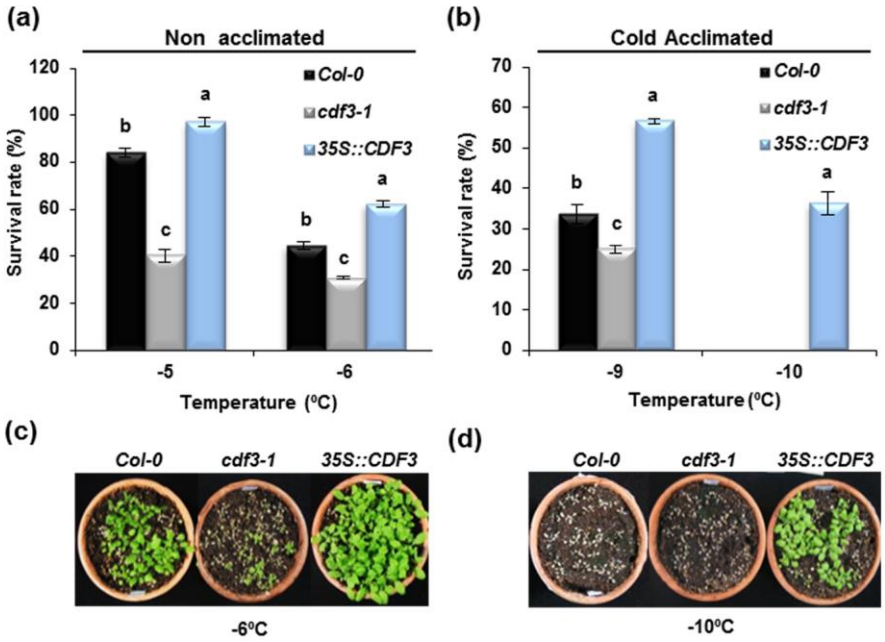

Figure 4. Effects of CDF3 on tolerance to low temperatures.

Figure. 4. Effects of CDF3 on tolerance of low temperatures.

(a-b) Freezing tolerance of nonacclimated (a) and cold acclimated (7 days at 4°C) (b) 2-week-old Col-0, *35S::CDF3* and *cdf3-1* plants that were exposed to the indicated freezing temperatures for 6h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7d of recovery under control conditions. Data are expressed as means ± SE of the three independent experiments with 50 plants each. Letters indicate significant differences between Col-0, *cdf3-1* and *35S::CDF3*; P<0.05 ANOVA followed by Student-Newman-Keuls test).

(c-d) Phenotypes of nonacclimated (c) and cold acclimated (d) Col-0, mutant and overexpressor plants after 7d of recovery after being exposed to the indicated freezing temperatures.

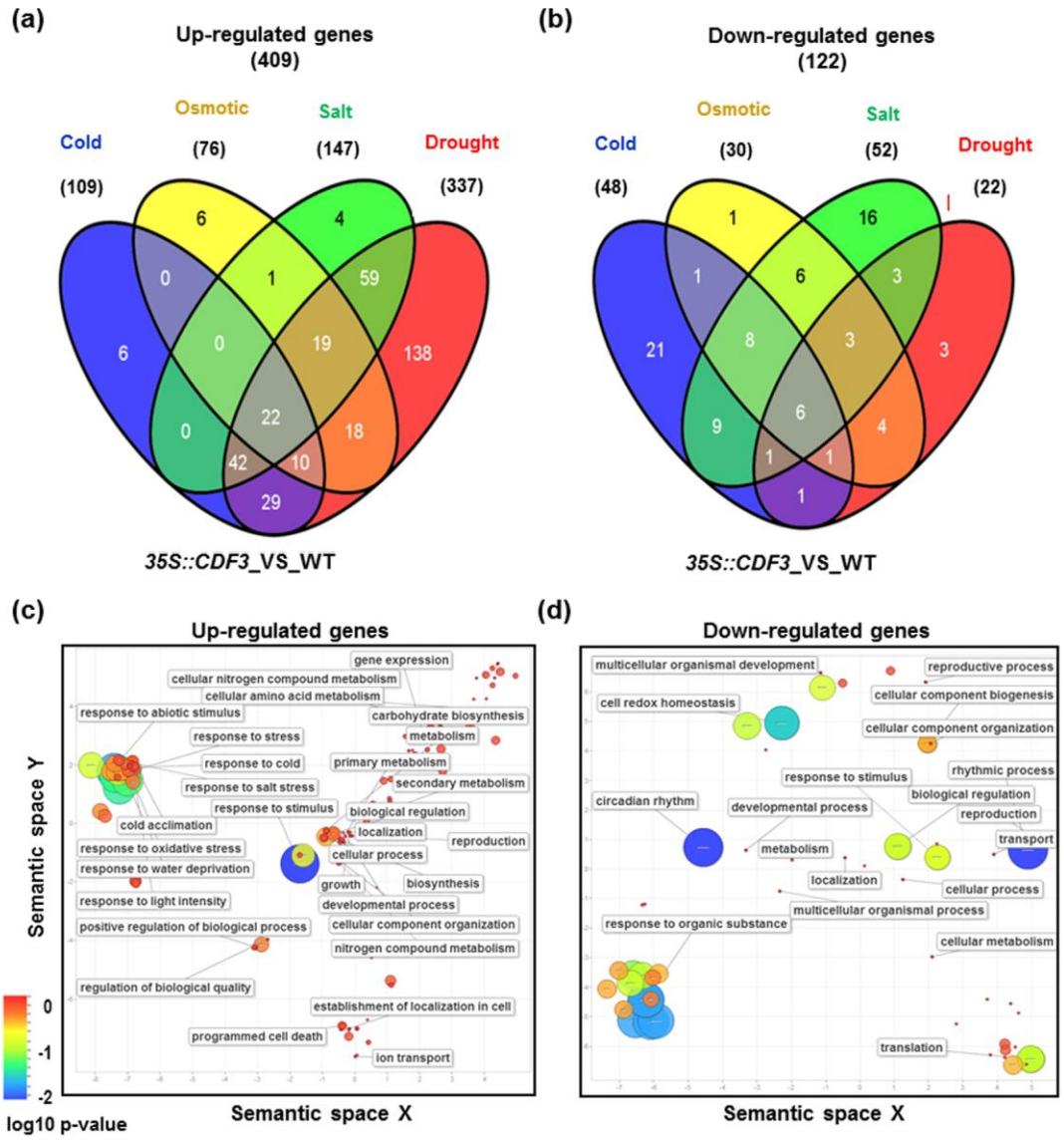

Figure. 5. Classification and Gene Ontology analyses of the genes differentially expressed in CDF3 overexpressing lines compared with WT plants.

Figure 5. Classification and Gene Ontology analyses of the genes differentially expressed in *CDF3* overexpressing lines compared with WT plants.

(a-b) Venn diagrams showing overlap of (a) up-regulated and (b) down-regulated genes expressed in *35S::CDF3* transgenic plants compared with WT plants in response to different stresses. *In silico* expression analyses and classification of

35S::CDF3 up-regulated and down regulated genes in response to cold, osmotic, salt and drought stresses, by using e-Northern Expression Browser tool.

(c-d) Scatter plot of (c) up-regulated and (d) down-regulated genes expressed in *35S::CDF3* compared with WT plants shows the cluster representatives (terms remaining after reducing redundancy) in a two-dimensional space. The scatter plots were performed using AgriGO and Revigo tools. Bubble color indicates the p-value for the false discovery rates derived from the AgriGO analysis as well as biological processes.

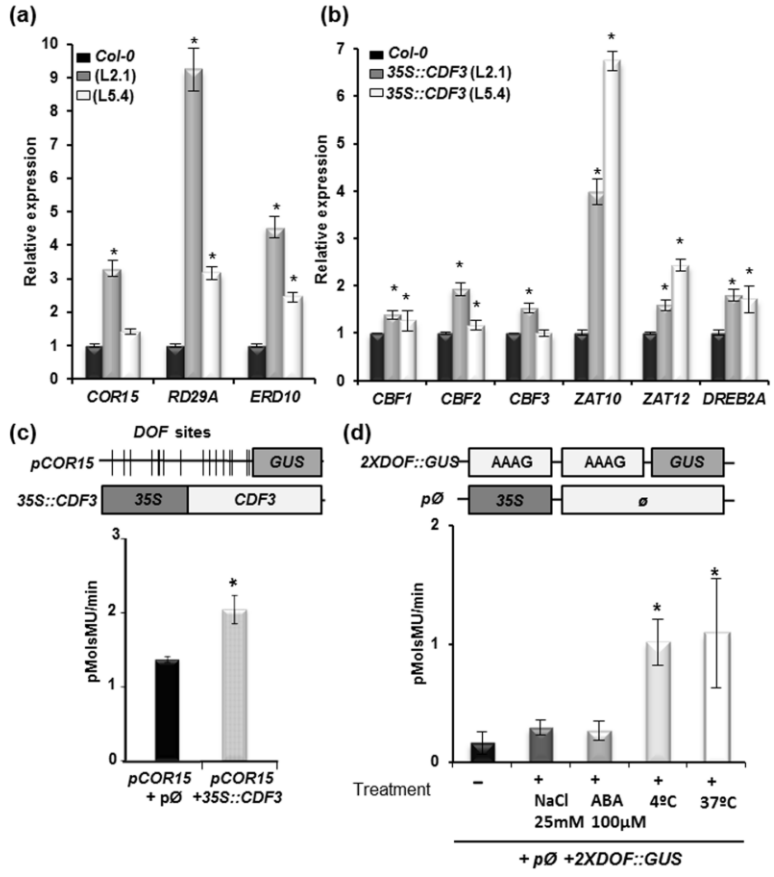

Figure 6. CDF3 regulates a set of genes involved in cellular osmoprotection and stress-related transcription factors.

Figure 6. CDF3 regulates a set of genes involved in cellular osmoprotection and stress-related transcription factors.

(a-b) Transcription analysis by qRT-PCR of *COR15*, *RD29A* and *ERD10* stress-responsive genes (a) and *CBF1, CBF2, CBF3, ZAT10, ZAT12* and *DREB2A* genes (b) in 4-week-old *35S::CDF3* (L2.1 and L5.4) and Col-0 plants. *UBIQUITIN21* gene was used as a reference gene. Data are means of ± SE (n=5). Asterisks indicate significant differences from WT; * P<0.05 by Student´s t-test.

(c) Transcriptional activation assay of *COR15* gene promoter by CDF3*. Arabidopsis* protoplasts were co-transfected with *pCOR15::GUS* reporter plasmid and effector *35S::CDF3* constructs. Empty effector plasmid was used as negative control. Data

are means ± SE (n=5). Asterisks indicate significant differences from control; * P<0.05 by Student´s t-test.

(d) DOF DNA motif is an abiotic stress-responsive element. Protoplasts were co-transfected with *pBT10-2xDOF-GUS* reporter plasmid and empty effector plasmid and exposed to control conditions (Control) or treated with NaCl (25mM) and ABA (100µM), or incubated at extreme temperatures (4ºC and 37ºC) for 12h. Data are means ± SE (n=3). Asterisks indicate significant differences from Control; * P<0.05 by Student´s t-test.

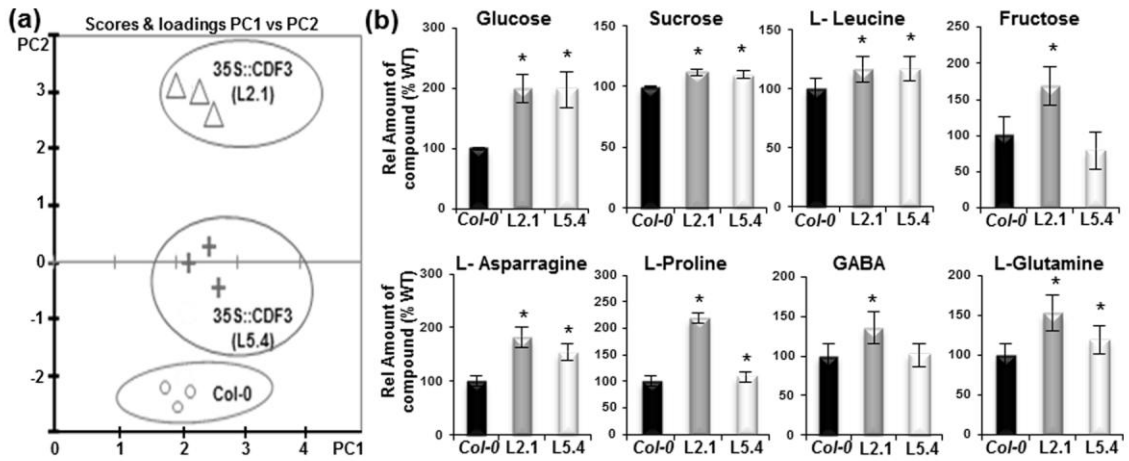

Figure 7. Effect of *CDF3* in sugar and amino acid metabolism.

Figure 7. Effect of CDF3 in sugar and amino acid metabolism.

(a) PCAs of recorded, non-targeted metabolic profiles. Projection plots are shown for principal component 1 (PC1, 28% variance explained) and PC2 (55.3%). Distinct grouping supports the different genotypes analyzed: Col-0 or overexpression lines 2.1 and 5.4, respectively.

(b) Relative quantities (% of WT) of selected metabolites analyzed by Gas chromatography-selected ion monitoring-mass spectrometry. Results are shown as means ±SE (n=15). Similar results were obtained in five independent experiments; * P<0.01; ANOVA, followed by a Student-Newman Keuls test.