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RESEARCH PAPER

Characterization of tomato Cycling Dof Factors reveals conserved and new functions in the control of flowering time and abiotic stress responses

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Abstract

DNA binding with One Finger (DOF) transcription factors are involved in multiple aspects of plant growth and development but their precise roles in abiotic stress tolerance are largely unknown. Here we report a group of five tomato DOF genes, homologous to *Arabidopsis* Cycling DOF Factors (CDFs), that function as transcriptional regulators involved in responses to drought and salt stress and flowering-time control in a gene-specific manner. SlCDF1–5 are nuclear proteins that display specific binding with different affinities to canonical DNA target sequences and present diverse transcriptional activation capacities *in vivo*. *SlCDF1–5* genes exhibited distinct diurnal expression patterns and were differentially induced in response to osmotic, salt, heat, and low-temperature stresses. *Arabidopsis* plants overexpressing *SlCDF1* or *SlCDF3* showed increased drought and salt tolerance. In addition, the expression of various stress-responsive genes, such as *COR15*, *RD29A*, and *RD10*, were differentially activated in the overexpressing lines. Interestingly, overexpression in *Arabidopsis* of *SlCDF3* but not *SlCDF1* promotes late flowering through modulation of the expression of flowering control genes such as *CO* and *FT.* Overall, our data connect SlCDFs to undescribed functions related to abiotic stress tolerance and flowering time through the regulation of specific target genes and an increase in particular metabolites.

Key words: CDF, DOF, drought stress, gene expression, flowering time, salt stress, tomato.

Introduction

DNA binding with One Finger (DOF) proteins are a group of plant-specific transcription factors (TFs) that contain a 50 aa conserved domain in the N-terminal region. This DOF domain corresponds to a C2–C2 configured zinc finger that binds specifically to the 5′-T/AAAAG-3′ sequence motif in the promoters of direct target genes (Yanagisawa and Schmidt, 1999). In contrast, the C-terminal protein region has a highly variable structure, containing specific

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; ANOVA, analysis of variance; bZIP, basic leucine zipper; CDF, Cycling Dof Factors; DOF, DNA binding with One Finger; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; HMG, high-mobility group; LD, long day; LL, continuous light; ORF, open reading frame; PEG, polyethylene glycol; PCA, principal component analysis; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; TF, transcription factor.

protein–protein interaction domains and other regulatory elements. For instance, the Thr-Met-Asp motif present in *Arabidopsis* AtDOF4.2 and AtDOF4.4, (Zou *et al.*, 2013) and a 48 aa C-terminal domain of maize ZmDOF1 are responsible for their activation capacity (Yanagisawa and Sheen, 1998; Yanagisawa, 2001). Consequently, DOF TFs exhibit a complex modular structure, which allows them to display multiple regulatory functions, acting both as activators or repressors in control of the expression of numerous plant genes (Mena *et al.*, 1998; Yanagisawa and Sheen, 1998; Diaz *et al.*, 2002; Yamamoto *et al.*, 2006). The regulatory activity mediated by DOF proteins involves not only DNA binding to target sequences but also specific protein–protein interactions with other regulatory proteins including basic leucine zipper (bZIP) and MYB TFs (Zhang *et al.*, 1995; Vicente-Carbajosa *et al.*, 1997; Washio, 2001; Diaz *et al.*, 2002) and nuclear high-mobility group (HMG) proteins (Yanagisawa, 1997; Krohn *et al.*, 2002).

Over the last years, DOF proteins have been reported to contribute to the control of very different biological processes, as diverse as seed maturation and germination, tissue-specific gene expression, light responses, and plant hormone signalling (Yanagisawa, 2002, 2004; Moreno-Risueno *et al.*, 2007*a*, *b*). DOFs participate in the control of genes involved in carbon fixation and nitrogen assimilation (Yanagisawa and Sheen, 1998; Rueda-López *et al.*, 2008), secondary metabolism (Skirycz *et al.*, 2006, 2007), vascular development (Konishi and Yanagisawa, 2007; Guo *et al.*, 2009; Gardiner *et al.*, 2010), lipid metabolism in the seed (Wang *et al.*, 2007), seed germination (Papi *et al.*, 2000, 2002; Gualberti *et al.*, 2002), photoperiodic flowering (Imaizumi *et al.*, 2005; Iwamoto *et al.*, 2009), and flower abscission (Wei *et al.*, 2010). Nevertheless, *DOF* genes involvement in the regulation/adjustment of the metabolism under different environmental cues has not been described.

The family of DOF TFs evolved from a common ancestor in green unicellular algae such as *Chlamydomonas reinhardtii*, where only one gene has been found, and rapidly expanded in mosses, ferns, and vascular plants (Moreno-Risueno *et al.*, 2007*a*). *DOF* genes are classified into families of different size within species. *In silico* analyses of the complete genome sequences of *Arabidopsis*, rice, and *Brachypodium* predicted 36, 30, and 27 *DOF* genes, respectively (Lijavetzky *et al.*, 2003; Hernando-Amado *et al.*, 2012), whereas 31 members have been found in wheat (Shaw *et al.*, 2009), 26 in barley (Moreno-Risueno *et al.*, 2007*a*), and 28 in sorghum (Kushwaha *et al.*, 2011). Different phylogenetic analyses using *Arabidopsis*, rice, barley, and *Brachypodium* sets of predicted *DOF* genes indicate that they can be classified into four major clusters of orthologous genes or subfamilies, A–D (Lijavetzky *et al.*, 2003; Hernando-Amado *et al.*, 2012). In *Arabidopsis*, the D group contains a set of DOF factors whose transcripts oscillate under constant light conditions and are hence known as Cycling Dof Factors, CDF1–5 (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). CDFs display an important role in photoperiodic flowering in *Arabidopsis* through the establishment of a diurnal rhythm in *CONSTANS* (*CO*) transcript levels by repressing its expression. When overexpressed, *CDF1–5* repress *CO* transcription, causing a strong delay of flowering under long-day (LD) conditions. Consistently, combining loss-of-function alleles in four of these genes (*CDF1*, *2*, *3*, and *5*) causes photoperiod-insensitive early flowering (Fornara *et al.*, 2009). *In vivo*, CDF1 and CDF2 degradation depends of the action of a protein complex that includes FLAVIN-BINDING KELCH REPEAT F-BOX PORTEIN (FKF1) and GIGANTEA (GI) (Sawa *et al.*, 2007). Light is required to stabilize their interaction, so longer photoperiods cause enhanced accumulation of GI–FKF complexes and consequently decreased CDF protein levels (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009).

The Solanaceae family includes several horticultural crops of major economic importance, such as tomato, potato, tobacco, and pepper. Although wide tolerance levels to abiotic stresses can be found in their wild relative species, only moderate tolerance is conserved among their cultured varieties (Shannon and Grieve, 1999; Nuez and Prohens, 2008). In the case of tomato, most cultivars show negative effects under drought and salinity, resulting in growth inhibition, decreased seed germination, and reduction of fruit quality and production (Cuartero *et al.*, 1995; Cuartero and Fernández-Muñoz, 1999). At the molecular level, abiotic stresses induce changes in the expression of a large number of genes, leading to physiological and biochemical alterations. Drought and salinity significantly affect photosynthesis, which impacts on the function of other important metabolic pathways such as nitrogen assimilation (Chaves *et al.*, 2009). Moreover, respiration is enhanced to provide energy to maintain plant growth and development (Haupt-Herting *et al.*, 2001). Other protection systems are also affected by drought and salt stress, such as the antioxidant and osmoregulation pathways that reinforce plant cells by the biosynthesis of compatible solutes and reactive oxygen species (ROS) scavengers (Blumwald, 2000; Zhu, 2001, 2003; Apel and Hirt, 2004; Munns and Tester, 2008).

Some efforts in the identification of genes responsible for salt and drought tolerance have been made for both wild and cultivated tomato plants. Recent global expression analyses showed that more than 2000 and 1300 genes are induced or repressed in response to drought and salinity, respectively (Gong *et al.*, 2010; Sun *et al.*, 2010), suggesting that responses to these stresses are mediated by multiple signal transduction pathways. Moreover, a number of the identified genes are commonly affected by both stresses and by different stress conditions such as low and high temperatures (Gong *et al.*, 2010; Sun *et al.*, 2010), indicating an overlap of plant responses to abiotic stress. Despite these efforts, only a small number of transcriptional regulators have been demonstrated to participate in abiotic stress responses in the Solanaceae, like LebZIP2 (Seong *et al.*, 2008), SlAREB1 (Yáñez *et al.*, 2009), SlAREB1 (Orellana *et al.*, 2010) StERBEP1 (Lee *et al.*, 2007), AIM1 (Abuqamar *et al.*, 2009), TERF1 (Huang *et al.*, 2004), and JERF1 (Wu *et al.*, 2007).

Expression levels of certain *DOF* genes are regulated by several environmental conditions. Nevertheless, especially in crop plants like tomato, their exact roles in abiotic stress tolerance are not known. In this work, we identified 34 DOFs in tomato and performed phylogenetic analyses and

comparisons with their *Arabidopsis* counterparts. Based on sequence similarity and domain analyses, we identified five genes homologous to *Arabidopsis CDF*s. We explored their expression patterns during plant development, in response to abiotic stresses, and under different light conditions. Among them, *SlCDF1* and *SlCDF3* were investigated in more detail, focusing particularly on their roles in photoperiodic flowering response and abiotic stress tolerance. *Arabidopsis* plants overexpressing *SlCDF1* and *SlCDF3* genes showed improved tolerance to drought and salt when compared with the wild type (WT). Combined studies of putative downstream target genes and metabolite profiling shed light on the molecular basis of the uncovered new roles of CDF proteins in response to environmental stresses.

Material and methods

Database searches for identification of DOF family members in Solanum lycopersicum

The nucleotide DOF domain sequences of *Arabidopsis CDF* genes (Lijavetzky *et al.*, 2003) were used to search for potential *DOF* genes in the tomato genome using the BLAST program (Altschul *et al.*, 1997) at the Sol Genomics Network website (Bombarely *et al.*, 2011) and Phytozome database (Goodstein *et al.*, 2012). The amino acid sequences of the *DOF* genes were deduced through the 'Translate tool' at ExPASy Proteomics Server (Artimo *et al.*, 2012). Alignments of protein sequences were performed by CLUSTALW (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA program software version 5.0 (Guindon and Gascuel, 2003; Tamura *et al.*, 2011), obtaining the phylogenetic trees from neighbour-joining analysis. The deduced protein sequences of CDFs from tomato and *Arabidopsis* were analysed further by means of the MEME program (Bailey *et al.*, 2009; http://meme.sdsc.edu/meme4_6_0/intro.html).

Subcellular localization of tomato CDF proteins

Open reading frames (ORFs) of the tomato *SlCDF* genes were cloned into the pK7WGF2.0 plasmid using the Gateway recombination system (Invitrogen) to generate C-terminal green fluorescent protein (GFP) fusions driven by the cauliflower mosaic virus (CaMV) 35S promoter (Karimi *et al.*, 2007). As a control, the *GFP* gene expressed under the control of 35S promoter was used. Transient transformations of onion (*Allium cepa* L.) epidermal cells were performed by particle bombardment with a biolistic helium gun device (DuPont PDS-1000; Bio-Rad) as described by Diaz *et al.* (2002). Fluorescence images were acquired after 40 h of incubation at 22 °C in the dark using a confocal microscope (LEICA Sp2 AOBS UV) with appropriate filters.

DNA-binding specificity of CDF proteins using a yeast one-hybrid assay

Two copies of the DOF *cis*-DNA element were produced by annealing the complementary single-stranded oligonucleotides pTUYDOF-S (5′-CGT GACATGTAAAG TGAATAACGTGACA TGTAAAG TGAATAA-3′) and pTUYDOF-AS (5′-CTAG TTATTCACTTTA CATGTCACGT TATTCACTTTACATGT CACGAGCT-3′), which generated *Xma*I and *Xba* I cohesive ends. This fragment was cloned into the *Xma*I and *Xba*I sites of the reporter plasmid pTUY1H (Clontech) containing the *HIS3* nutritional reporter gene. Entry clones containing the ORFs of the *SlCDF1–5* genes were recombined into the pDEST22 plasmid (Invitrogen) using the LR reaction to generate GAL4AD–ORF fusions. The resultant constructs and pTUY1H-2×DOF were co-transfected into HF7c yeast cells. As a negative control, an empty pDEST22 and pTUY1H-2×DOF vectors were used. Transformed yeast cells were plated onto SD/–Trp–Leu medium and incubated at 28 °C. Single colonies were then streaked on SD/–Trp–Leu–His selection medium with 30 mM 3-amino-1,2,4-triazole (3-AT). The plates were subsequently incubated at 28 °C for 2 d and yeast growth was determined.

Protoplast transformation and GUS assays

Mesophyll protoplasts were isolated from rosette leaves of 3-week-old *Arabidopsis* plants ecotype Columbia (Col-0) grown in soil (21/18 °C, 8/16 h light/dark). Protoplast isolation and transfection were performed according to the method described by Alonso *et al.* (2009). Plasmid DNA was prepared using a Genopure Plasmid Maxi kit (Roche), and 5 µg of pBT10-2×DOF-*GUS* (a dimer of the DOF binding element) and 14 µg of each *SlCDF1–5* effector plasmid were used for transfections. For normalization purposes, 1 µg of Pro_{35S}::*NAN* plasmid (Kirby and Kavanagh, 2002) was added. Then, 20 µl of plasmid mixture (20 µg) and 200 µl of protoplasts were transferred to 2 ml microcentrifuge tubes following the procedure described by Weltmeier *et al.* (2006). β-Glucuronidase (GUS) and NAN enzyme assays were performed according to Kirby and Kavanagh (2002). The ratio of GUS and NAN activities are represented as relative GUS/NAN units.

Plant growth conditions and quantification of CDF *gene expression in tomato*

Characterization of the expression of *CDF* genes in tomato was performed in the Marmande RAF cultivar. Seeds were germinated on a moistened mixture of peat moss and sand in growth chambers (25/20 °C, 16/8 h photoperiod) and irrigated regularly with alternating water and nutrient solution (Hoagland and Arnon, 1950). To study the expression profiling of *SlCDF* genes during vegetative and reproductive development, we collected plant material at different developmental stages: imbibed seeds, radicles, and cotyledons from 3-d-old seedlings; roots and leaves from 30-d-old plants; roots, leaves, and flowers (in anthesis) from 60-d-old plants; and green (30 d after anthesis) and red (60 d after anthesis) fruit mesocarp. Three different pools of each plant material were harvested at any developmental stage. To study the effect of abiotic stress and light regulation on the expression of *SlCDFs*, 3-week-old uniform plantlets, bearing three leaves, were transferred to 1 l plastic pots containing half-strength Hoagland solution. Solutions were aerated and replaced every 4 d, and plants were maintained for 4 weeks in growth chambers (25/20 °C; 16/8 h photoperiod). Salt stress was assayed by adding NaCl at 50 mM in the nutrient solution. Polyethylene glycol (PEG 8000; Sigma) at 5% was used for water stress. Plants were transferred for 24 h to growth chambers at 35/30 and 10/5 °C, for high- and low-temperature stresses, respectively. Three different pools of roots and leaves were harvested (four plants per pool) after 6, 12, and 24 h of initiating the stress. Control plants were maintained at 25/20 °C in half-strength nutrient solution. To study the diurnal changes in the expression of *SlCDF* genes, leaves were harvested at 6 h intervals for a total of 24. For continuous light (LL) experiments, plants were shifted to continuous light at dawn. After 24 h, leaves were harvested every 4 h for 24 h (0, 4, 8, 12, 16, 20, and 24 h). Three independent extracts, obtained from 12 plants (two leaves per plant and four plants per extract) were assayed at the different time points in both experiments. Plant material was collected and stored at –80 °C until analysed. Total RNA was extracted and purified using an RNeasy Mini kit (Qiagen) and treated with Turbo DNase (Ambion) following the manufacturer's protocol. cDNA was synthesized from 2 µg of DNA-free RNA with the use of Superscript II reverse transcriptase (Invitrogen) and random hexamers. An ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used for real-time PCR with programs recommended by the manufacturer (2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min)

using Power SYBR Green PCR master mix (Applied Biosystems). In all treatments and conditions, three independent samples from different extracts were used and each reaction was performed in triplicate. The primer pairs used for amplification are described in Supplementary Table S3 available at *JXB* online. The *UBIQUITIN3* gene from *S. lycopersicum* (Hoffman *et al.*, 1991) was used as reference gene. Relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Positive and negative controls were included in the quantitative real-time PCR (qRT-PCR) analyses.

Plasmid constructs and plant transformation

The ORFs of *SlCDF1* and *SlCDF3* were cloned into the Gateway binary vector pGWB2 (Nakagawa *et al.*, 2007) under the control of the 35S promoter. The resultant plasmid was used to transform *Arabidopsis thaliana* (Col-0) plants by the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). Transformed plants were selected on MS medium (Murashige and Skoog, 1962) containing 50 µg ml^{-1} of kanamycin.

RNA measurements by qRT-PCR in Arabidopsis

The expression of *SlCDF* genes (*SlCDF1* and *SlCDF3*), abiotic stress-responsive genes (*COR15*, *RD29A*, and *ERD10*), and flowering control genes (*CO* and *FT*) in overexpression (*35S::SlCDF1* and *35S::SlCDF3*) and control lines (Col-0) was determined by qRT-PCR. Plants were maintained in growth chambers (21/18 °C, 16/8 h photoperiod). Total RNA was extracted from 10-d-old seedlings to study *CO* and *FT* expression and from leaves of 3-week-old plants to study *SlCDF1-3*, *COR15*, *RD29A*, and *ERD10* following the protocol of Oñate-Sánchez and Vicente-Carbajosa (2008). For cDNA synthesis, 2 µg of total RNA was primed with oligo$(dT)_{15}$ primers (Promega) using the avian myeloblastosis virus reverse transcriptase according to the manufacturer's instructions. *Arabidopsis UBIQUITIN* mRNA level (At5g25760) was used as a control. The reaction, PCR program, and analysis of the data were performed as described above to analyse the expression of *CDF* genes in tomato. The primers pairs used for PCR amplification are given in Supplementary Table S3, available at *JXB* online.

Salt and drought stress tolerance tests

Salinity and drought stress assays were carried out using control plants (Col-0) and *35S::SlCDF1* and *35S::SlCDF3* transgenic lines. For salinity assays, seeds were sterilized and plated onto Petri dishes containing MS medium. After 6 d, the seedlings were transferred to vertical plates containing MS medium (control) and MS medium supplemented with 80 mM NaCl (Lakhssassi *et al.*, 2012). About 20 seedlings were used per replicate and three replicates were made for each treatment. Primary and lateral root elongation was measured after 10 d using ImageJ software (Abràmoff *et al.*, 2004). To evaluate growth differences between control and saline stress, data were represented as the percentage of root growth reduction relative to standard conditions. Statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test ($P<0.01$). Drought stress tolerance tests were performed on plants grown in soil in individual pots. After 2 weeks, the water supply was cut off for 15 d and watering was then resumed for 10 d. Plant survival rates were calculated afterwards and fresh weight was measured 10 d after the rewatering period.

Metabolomic analyses

Non-targeted and targeted metabolomics analyses were performed on 12-d-old control plants (Col-0) and two independent *35S::SlCDF3* lines. Extraction, manipulation, and mass spectrometric analysis of samples followed an adapted protocol, detailed in Supplementary File S1 (available at *JXB* online), which is based on previously described methods (Fiehn *et al.*, 2000; Gullberg *et al.*, 2004; Gaquerel *et al.*, 2010).

Results

Identification of CDF proteins in tomato plants

In order to identify CDF proteins encoded by the tomato genome, the amino acid sequence of the DNA-binding domain of *Arabidopsis* CDF1–5 proteins (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009) was used to perform a BLAST survey against the tomato whole-genome database (http://solgenomics.net/; Bombarely *et al.*, 2011). A total of 34 predicted *DOF* tomato transcription factor genes were identified and annotated, and were named *SlDOF1–34* (*S. lycopersicum DOFs*; Supplementary Table S1 available at *JXB* online). Nucleotide sequence comparisons between genomic and cDNA clones allowed the identification of precise exon–intron structures (Supplementary Table S2 available at *JXB* online). All encoded DOF proteins contained a unique DNA-binding domain of 50 aa encompassing a C2–C2 zinc finger (DOF). In a previous study, Lijavetzky *et al.* (2003) identified 36 DOF proteins in *Arabidopsis* and classified them into four groups, A–D. In order to evaluate the evolutionary relationships among the tomato and *Arabidopsis* DOFs, specific and combined phylogenetic analysis based on their DNA-binding domain sequences were performed. The resulting trees were obtained by the neighbour-joining algorithm and supported by comparisons with the *Arabidopsis* tree (Fig. 1A, B, Supplementary Fig. S1 available at *JXB* online). In both species, DOFs were clustered into four mayor groups: A, B, C, and D. Three of them were further divided into subgroups based on bootstrapping values. *Arabidopsis* group D1 contained the *Arabidopsis CDF*s, i.e At5g62430, At5g39660, At3g47500, At1g26790, and At1g69570. Interestingly, sequence analyses also identified a D-type group in tomato, containing five genes encoding proteins with a high level of sequence similarity to the *Arabidopsis* CDFs. These tomato genes were considered to be putative *CDF* orthologues from tomato and were renamed as *S. lycopersicum CDF1–5* (Supplementary Table S1, available at *JXB* online). This tentative assignation was further supported by comparative analyses of the deduced amino acid sequences of the whole *Arabidopsis* and tomato CDF proteins by the MEME software. As shown in Fig. 1C, the analyses revealed the existence of homologous motifs, conserved among their sequences and different from the DOF binding domain characteristic of this family (motif 1; Lijavetzky *et al.*, 2003; Yanagisawa 2004; Moreno-Risueno *et al.*, 2007a). Two additional conserved domains are also found in all of the proteins: motifs 2 and 4 spanning 21 and 22 aa, respectively; and another 33 aa motif (motif 3) conserved in nine of 10 sequences. These three associated motifs seemed to represent a common signature of type D group of CDF proteins of *Arabidopsis* and tomato.

Tomato SlCDF1–5 proteins localize to the cell nucleus and display distinct DNA-binding and activation properties

To investigate the subcellular localization of SlCDF proteins, translational fusions of their corresponding ORFs to the C terminus of GFP were made. These constructs, driven by the

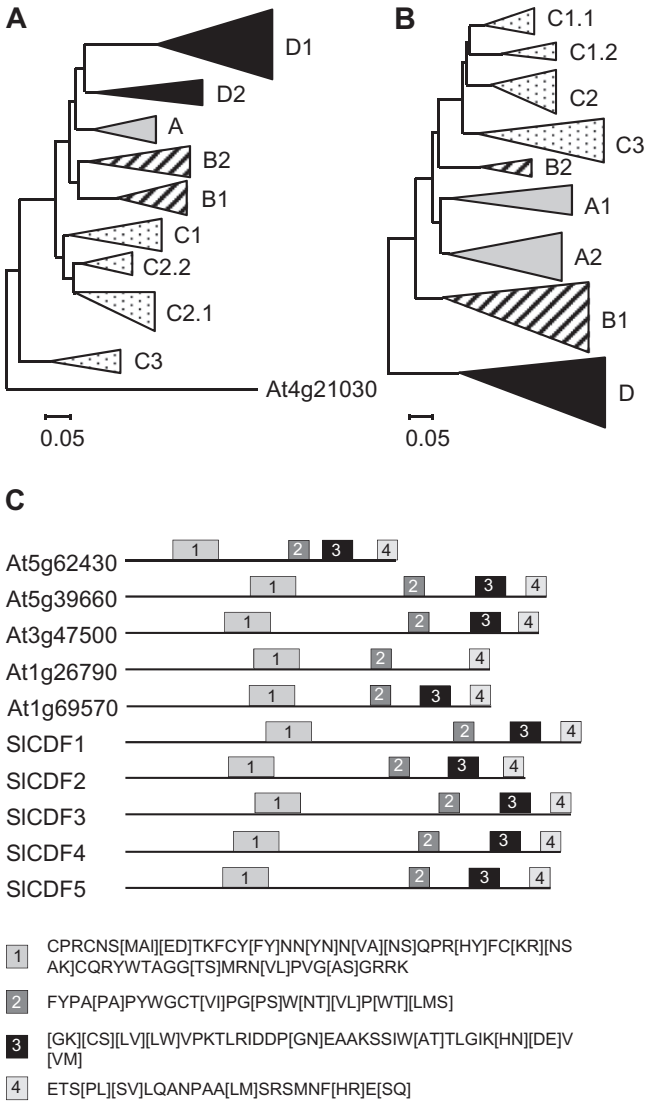

Fig. 1. Phylogenetic trees and conserved motifs of *Arabidopsis* and tomato DOF protein families. (A, B) The *Arabidopsis* (left) and tomato (right) trees were inferred by the neighbour-joining method after alignment of the DOF domain amino acid sequences of the 36 *Arabidopsis* (Lijavetzky *et al.*, 2003) and 34 tomato DOF proteins (listed in Supplementary Table S1, available at *JXB* online), respectively. The resulting groups are shown as A, B, C, or D, and numbers indicate defined subgroups. Bar, 0.05 estimated amino acid substitutions per site. (C) Schematic distribution of conserved motifs among *Arabidopsis* and tomato CDF proteins. Motifs were identified by means by MEME software using the complete amino acid sequences of the 10 CDF proteins clustered in group D of the phylogenetic trees. The position of the identified motifs is relative to the DOF domain. Multilevel consensus sequences for the MEME-defined motifs are listed.

35S promoter, were used in transient assays with onion epidermal cells by particle bombardment. As shown in Fig. 2A, fluorescence corresponding to the emission spectrum of GFP was restricted to the nuclei of transformed cells that carried the *35S::GFP::SlCDF* constructs (Fig. 2A, panels 8–12). When cells were transiently transformed with *35S::GFP*, the GFP fluorescence was spread throughout the cell, indicating a cytoplasmic localization (Fig. 2A, panel 7). Nomarski pictures (Fig. 2A, panels 1–6) and the merged pictures of these and the fluorescence images are also shown (Fig. 2A, panels 13–18). We examined the capacity of the tomato SlCDF proteins for binding to the 5′-AAAG-3′ *cis*-DNA element using the yeast one-hybrid system. Fig. 2B shows the results of an experiment where the different SlCDFs were expressed as fusion proteins to the GAL4 activation domain in yeast cells harbouring a *HIS3* reporter gene under control of a minimal promoter containing a 2×DOF *cis*-DNA element. Yeast growth on His-depleted medium results from activation of the *HIS3* gene through binding of the SlCDF proteins to the *cis*-DNA element. Addition of 3-AT as an inhibitor of the *HIS3* product was used to measure the strength of the protein–DNA-mediated activation. In all cases, effective yeast growth demonstrated that SlCDF–DNA binding was sufficiently strong to overcome 3-AT inhibition. However, yeast cells expressing *SlCDF1*, *SlCDF2*, and *SlCDF5* grew much better on medium containing 30 mM of 3-AT than those expressing *SlCDF3* and *SlCDF4*, indicating their higher binding affinity to the 5′-AAAG-3′ motif than the latter.

In order to test the transcriptional activation properties of SlCDFs *in planta*, transient expression analyses in *Arabidopsis* protoplasts were performed (Fig. 2C). The *35S::SlCDF1–5* effector plasmids were co-transfected with reporter plasmid pBT10-GUS-2×DOF. The results confirmed that all of the tested CDFs could bind to the 5′-AAAG-3′ *cis*-DNA element, although to different extents, and activate the reporter gene. This showed that the previously detected DNA-binding capacity is fully functional in leaf protoplasts. Interestingly, high levels of GUS activity were observed in protoplasts transformed with *SlCDF3*, *SlCDF4*, and *SlCDF5*, whereas low levels were detected in those protoplasts that were transformed with *SlCDF1* and *SlCDF2*. Overall, the data obtained indicated that the identified tomato SlCDFs are functional nuclear factors that, despite their high sequence similarity, bind the DOF element with different affinities and display distinct transcriptional activation capacities.

Expression of tomato SlCDFs follows a circadian rhythm

To investigate whether the identified *SlCDF1–5* genes from tomato are controlled by the circadian clock as in *Arabidopsis* (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009), we performed qRT-PCR analyses using RNA from tomato plants grown under a LD diurnal cycle of 16 h/8 h light/dark and under continuous light (LL), respectively. The results revealed that, under LD conditions, the expression levels of tomato *SlCDF1–5* oscillated during the day, although they displayed quite different patterns, which could be classified in two groups (Fig. 3A, B). The expression levels of *SlCDF1* and *SlCDF3* followed a similar pattern that consisted of upregulated levels during the second half of the night and the first part of the day, reaching a maximum level at approximately midday. The expression levels then rapidly decreased to lower levels in the middle of the night (Fig. 3A). In contrast *SlCDF2*, *SlCDF4*, and *SlCDF5* transcript levels dropped during the first part

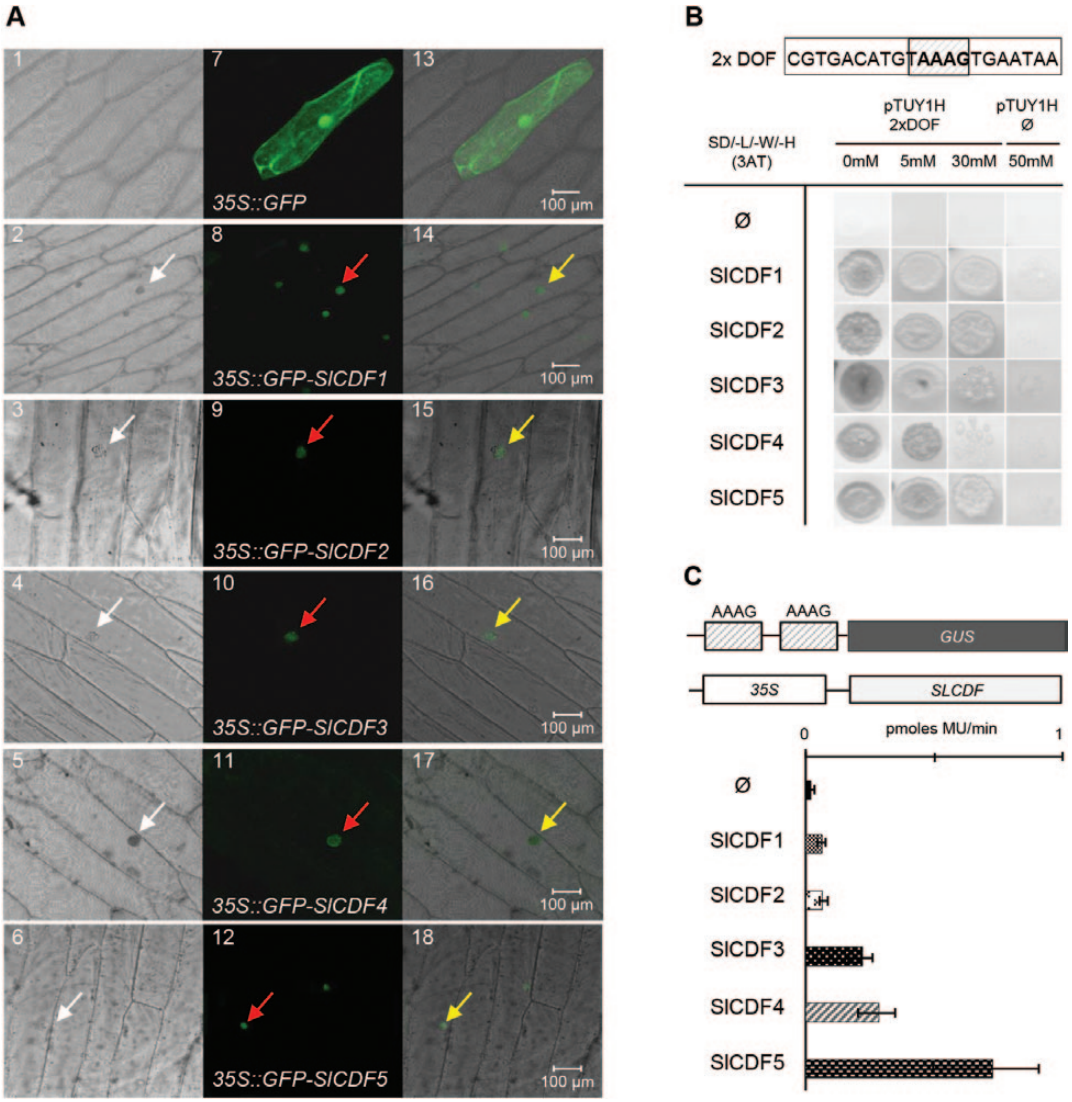

Fig. 2. Subcellular localization, transcriptional activation and DNA-binding specificity of tomato SlCDF1–5 proteins. (A) Subcellular localization of the SlCDF proteins in onion epidermal cells. GFP alone (35S::GFP) or GFP–SlCDF (35S::GFP-SlCDF1–5) fusion proteins were expressed transiently under the control of the CaMV 35S promoter in onion epidermal cells. After 36 h of incubation, tissues were observed with a confocal microscope for the emission spectrum of the GFP (panels 7–12) or by Nomarski imaging (1–6). Merged Nomarski and fluorescence images are also shown (panels 13–18). Arrows indicate cell nuclei. (B) The DNA-binding specificity of SlCDF1–5 proteins was assayed using a yeast one-hybrid system. Yeast HF7c cells were transfected with the genes encoding SlCDF proteins and pTUY1H driving *HIS3* expression under the control of the 2×DOF binding element. The transformed yeast cells were plated onto the SD/–His/–Trp/–Leu medium including the indicated amounts of 3-AT. Empty pDEST22 plasmid was used as a negative control. (C) Transcriptional activation assays of SlCDFs in *Arabidopsis* protoplasts. *Arabidopsis* protoplasts were transfected with the *35S::SlCDF1–5* effector plasmids (pK7WGF2.0) and pBT10-2XDOF-GUS reporter plasmid, containing the 2×DOF *cis*-DNA element. Empty pK7WGF2.0 plasmid was used as a negative control. Data are expressed as means ±standard error (SE) of three independent experiments.

of the light period. Minimum expression levels were maintained during the second half of the day and the beginning of the night and increased to reach a maximum at the beginning of the light period (Fig. 3A). However, when the analyses were performed with plants grown under LL conditions, the expression of tomato *SlCDF1–5* genes exhibited a 24 h period oscillation pattern, which was similar to that observed under LD conditions (Fig. 3B). Moreover, the expression patterns of *SlCDF1–5* could still be classified into the same two groups. Taken together, these data indicated that the expression of *SlCDF1–5* is light responsive and follows a circadian pattern, which strongly suggests that the identified tomato *CDF* genes are true orthologues of the *Arabidopsis* CDFs.

Expression of tomato SlCDF1–5 *genes is differentially regulated during development*

We analysed the expression patterns of tomato *SlCDF1–5* genes during plant development using qRT-PCR (Fig. 3C, D) and found that *SlCDF1–5* genes had distinct patterns of

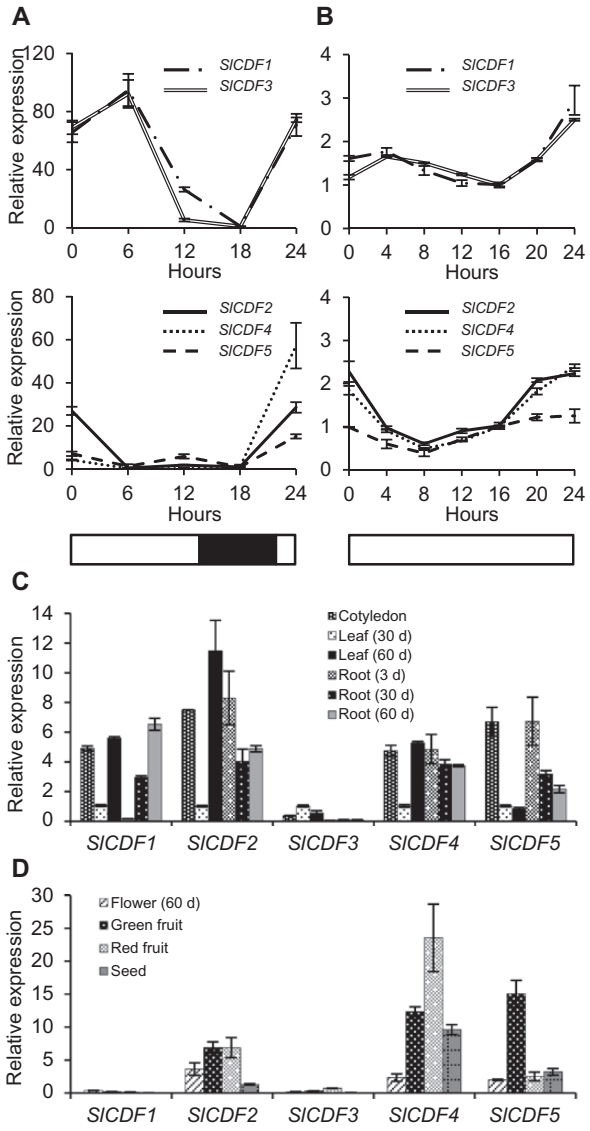

Fig. 3. Transcription analyses of tomato *SlCDF1–5* genes during development and in response to different light conditions. (A, B) *SlCDF1–5* gene expression analysed by qRT-PCR in 7-week-old tomato plants grown under a diurnal cycle of 16 h light/ 8 h dark or under continuous light. White and black bars along the horizontal axis represent light and dark periods, respectively. (C, D) Expression profiling of *SlCDF* genes. *SlCDF1–5* gene expression was analysed by qRT-PCR using RNA extracted from vegetative and reproductive tissues of tomato: radicles (root) and cotyledons from 3-d-old seedlings, root and leaves from 30- and 60-d-old plants, imbibed seeds, flowers from 60-d-old plants, and green and red fruit 30 and 60 d after anthesis, as indicated. Expression of the tomato *UBIQUITIN3* gene (Hoffman *et al.*, 1991) was used as a reference gene. All data are expressed as means ±SE of three independent pools of extracts. Three technical replicates were performed for each extract.

expression. *SlCDF1* and *SlCDF2* showed higher expression levels in vegetative compared with reproductive organs, while *SlCDF4* and *SlCDF5* were expressed at significant levels in both types. In addition, *SlCDF3* exhibited low expression in all organs analysed. The difference in expression patterns became more evident when the expression was analysed in closer detail during plant development (Fig. 3C). *SlCDF1*, *SlCDF2*, *SlCDF4*, and *SlCDF5* transcripts accumulated at high levels in cotyledons, but all showed minor levels of expression in mature leaves of 4-week-old plants. In contrast, a significant increment of *SlCDF1*, *SlCDF2*, and *SlCDF4* transcripts was detected in leaves of 8-week-old plants, while *SlCDF3* and *SlCDF5* showed a slight reduction. In addition, a progressive enhancement of *SlCDF1* expression was observed in roots during plant development. *SlCDF2*, *SlCDF4* and *SlCDF5* expression was, however, reduced in roots of older plants, and no changes were detected for *SlCDF3*. In the reproductive tissues analysed, the expression of *SlCDF1* and *SlCDF3* was negligible when compared with the other *SlCDF*s (Fig. 3D). Higher levels of *SlCDF2*, *SlCDF4* and *SlCDF5* transcripts were detected in flowers, fruits, and seeds. It is noteworthy that, during fruit ripening, a considerable increment in *SlCDF4* was detected, whereas *SlCDF5* transcripts were abundant only in green fruit, and *SlCDF2* showed similar expression in green and red fruit.

SlCDF1–5 *genes are differentially induced in response to abiotic stress conditions*

To address the question of whether the expression of *SlCDF*s is also regulated by environmental cues other than light/photoperiod, *SlCDF1–5* mRNAs levels were measured in leaves and roots of 3-week-old tomato plants that had been subjected to different abiotic stresses: salinity (50 mM NaCl), osmotic (5% PEG 8000), heat (35/30 °C) and cold (10/5 °C) treatments for 6, 12, and 24 h. In leaf tissues, transcript levels of all *SlCDF*s increased under salt and osmotic stress, in particular those of *SlCDF2* and *SlCDF4* after 24 h (Fig. 4A, B). In response to high temperatures, an earlier induction at 12 h was observed for *SlCDF4* and *SlCDF5*, with higher increases at 24 h together with *SlCDF2* (Fig. 4C). However, maximum induction was observed under cold treatment at 12 h for *SlCDF1*, *SlCDF3*, *SlCDF4*, and *SlCDF5*, with decay at 24 h (Fig. 4D). Induction of *SlCDF*s was also observed in root tissues following different patterns. All *SlCDF* genes were regulated by salt and drought. Most importantly, *SlCDF4* and *SlCDF5* showed induction after 24 h of salt treatment, whereas *SlCDF1*, *SlCDF2* and *SlCDF3* increased at early times (6 h) after osmotic treatment (Fig. 4A, B). Regarding temperature treatments, maximum increase was observed for *SlCDF3* and *SlCDF5* at 24 h after heat treatment (Fig. 4C), and for *SlCDF1*, *SlCDF3* and *SlCDF4* at 12 h after exposure to low temperatures (Fig. 4D).

Overexpression of tomato SlCDF3 *promotes late flowering in transgenic* Arabidopsis *plants*

Tomato *SlCDF1* and *SlCDF3* were selected for further characterization because they responded to various abiotic stresses and encoded proteins that showed the highest sequence similarity to the functionally well-characterized *Arabidopsis CDF1* (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). Transgenic

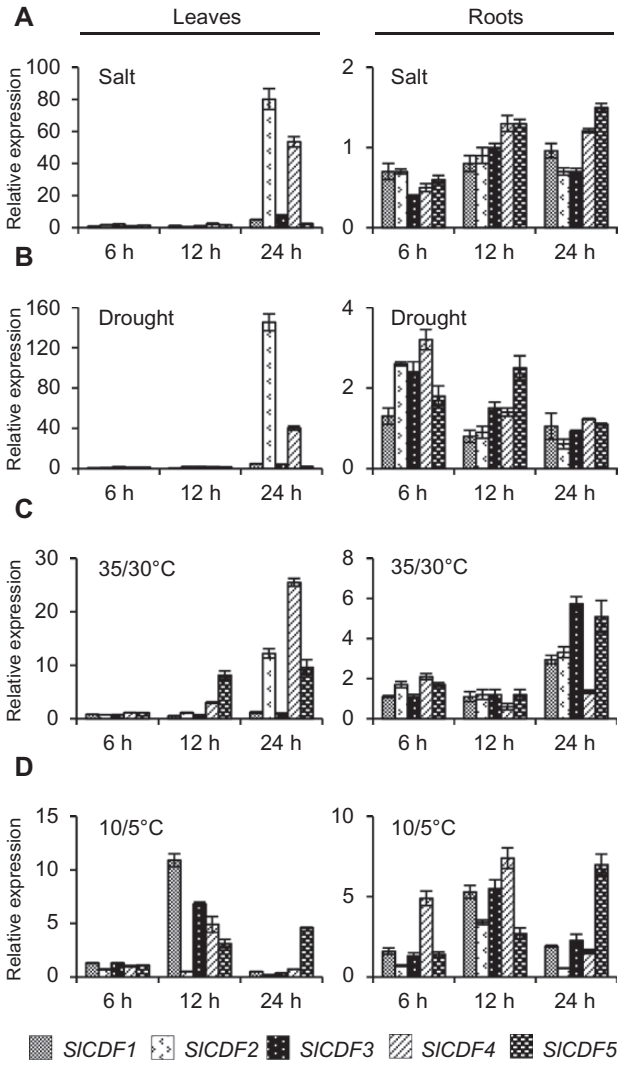

Fig. 4. Transcription analysis of tomato *SlCDF1–5* genes analysed by qRT-PCR in plants exposed to different abiotic stress conditions. Total RNA was extracted from 7-week-old tomato plants grown in nutrient solution (control) or supplemented with 50 mM NaCl for salt stress (A) or 5% PEG 8000 for drought stress (B), and exposed to 35/30 °C for high temperature stress (C) or 10/5 °C for low temperatures stress (D), for the indicated times. Expression of the tomato *UBIQUITIN3* gene (Hoffman *et al.*, 1991) was used as a reference gene. Results are presented as relative expression of *SlCDF1–5* under stress conditions compared with expression under control conditions. All data are expressed as means ±SE of three independent pools of extracts. Three technical replicates were performed for each extract.

Arabidopsis plants overexpressing *SlCDF1* and *SlCDF3* under the control of the CaMV 35S promoter were generated, and three homozygous lines with relatively high expression of *SlCDF1* and *SlCDF3* were selected for further analyses (see Fig. 7A). When cultured in soil under greenhouse conditions, all overexpressing *SlCDF3* lines (L2.10, L10.4, and L10.7) presented several developmental differences relative to WT plants (Col-0). Plants overexpressing *SlCDF3* flowered later than control plants under LD conditions but not under short-day conditions (Fig. 5A, B, C and J), suggesting that these plants are impaired in the photoperiodic flowering pathway. In addition, transgenic lines also displayed other pleiotropic alterations that became more evident in adult plants during both vegetative and reproductive development. Fig. 5D–H shows representative pictures of 4-week-old WT and *35S::SlCDF3* (line 10.7 as an example) plants showing that leaves were larger and petals and carpels of the mature flowers were larger than those of the WT. Furthermore, the siliques of the overexpressing lines were bigger than WT (Fig. 5I). In contrast, we did not observe significantly different phenotypes in the *SlCDF1*-overexpressing plants (data not shown). To assess whether the late-flowering phenotype observed in the *SlCDF3*-overexpressing plants was due to changes in the expression of reported key regulatory genes like *CO* and *FT*, we tested diurnal expression profiles of these genes by qRT-PCR, comparing *35S::SlCDF3* (L2.10 and L10.7) and WT plants. Fig. 6A shows that *CO* transcript levels decreased in the transgenic plants compared with the WT and the rhythmic cycling of the mRNA was dampened. Moreover, a reduction in the levels of *FT* expression was detected in *35S::SlCDF3* plants (Fig. 6A). Altogether, these data support the assumption that the tomato SlCDF3 exerts a similar mode of action as the *Arabidopsis* CDFs in the control of flowering time.

Overexpression of SlCDF1 *and* SlCDF3 *has an impact on drought and salt tolerance in transgenic* Arabidopsis *plants*

As our expression analyses indicated that tomato *SlCDF1* and *SlCDF3* might play an important role in the plant response to different abiotic stresses, we decided to further explore the function of SlCDF1 and SlCDF3. A phenotypic characterization of *35S::SlCDF1* and *35S::SlCDF3* plants was performed by analysing their response under abiotic stresses, such as dehydration and high-salt treatment. First, we studied the capacity of soil-grown *35S::SlCDF1* and *35S::SlCDF*3 transgenic plants to tolerate water deprivation compared with WT plants. After 15 d of drought, plants were allowed to recover for 10 d during which they were watered. As shown in Fig. 7B and C, when cultured in soil under non-stress (control) conditions, both WT and transgenic overexpressing lines performed equally well. After the drought treatment, all WT plants exhibited severe symptoms of water loss and substantial wilting. In contrast, most of the *35S::SlCDF1* and *35S::SlCDF3* transgenic plants were less affected, retaining greener leaves. Only slight wilting was observed in some of the *35S::SlCDF1* transgenic leaves. After the 10 d recovery period, the *35S::SlCDF1* and *35S::SlCDF3* transgenic plants exhibited better survival and growth than the WT, as judged by their survival rates and fresh weight (Fig. 7B, C). To assess tolerance to salt stress, primary and lateral root elongation assays were conducted. *35S::SlCDF1*, *35S::SlCDF3*, and WT plants were grown either on control medium (no NaCl) or on salt stress medium containing 80 mM NaCl for 10 d (Fig. 7D, E). Under control conditions, there was no difference between the transgenic and WT plants. Only two transgenic *35S::SlCDF3* lines (10.4 and 10.7) exhibited slightly longer roots. On salt stress medium,

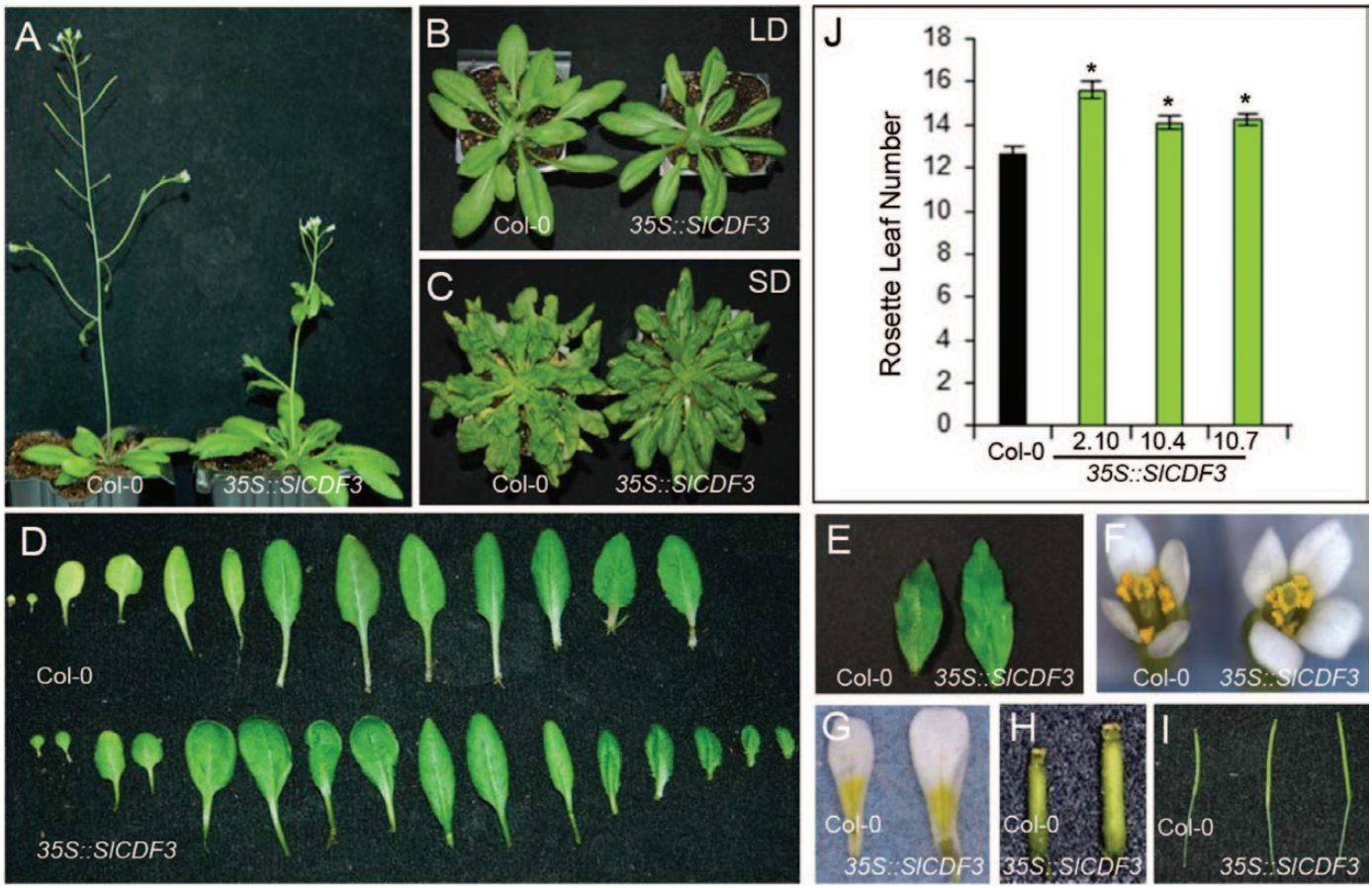

Fig. 5. Phenotypic differences in Col-0 and *35S::SlCDF3* plants during vegetative and reproductive development. (A) Representative images of 4-week-old WT and *35S::SlCDF3* (L10.7 as an example) plants grown under LD conditions. (B, C) Flowering-time phenotype under LD and short-day (SD) conditions, respectively. (D) Rossete leaves of Col-0 and *35::SlCDF3* plants grown under LD conditions. All leaves, including cotyledons, are shown in order of production from the first true leaf. (E) Cauline leaves of Col-0 and *35S::SlCDF3* plants grown under LD conditions. (F, G) Detached flowers and detached petals of Col-0 and *35S::SlCDF3* plants grown under LD conditions. (H) WT and *35S::SlCDF3* flower gynoecia. (I) Col-0 and *35S::SlCDF3* siliques. (J) Flowering-time analyses of Col-0 and *35S::SlCDF3* (L2.10, L10.4, and L10.7) lines estimated as rosette leaf number formed under LD conditions. Data are expressed as means ±SE of 20 homozygous plants. Asterisks indicate significant differences between Col-0 and *35S::SlCDF3*-overexpressing lines ($P<0.05$; one-way ANOVA, followed by a Student–Newman–Keuls test).

35S::SlCDF1 and *35S::SlCDF3* lines showed slight but significant lower values of primary growth inhibition than the WT. Moreover, the effect was more evident on lateral growth, as all *35S::SlCDF1* and *35S::SlCDF3* transgenic plants exhibited much lower values of lateral growth inhibition than WT plants under similar stress conditions (Fig. 7D, E). Collectively, these data suggested that SlCDF1 and SlCDF3 may be involved in plant responses to drought and salt stress.

To investigate the molecular mechanisms underlying the enhanced tolerance to drought and salt tolerance by SlCDF1 and SlCDF3, we tested the expression levels of different abiotic stress-responsive genes such as *COR15A*, *RD29A*, and *ERD10* in *35S::SlCDF1*, *35S::SlCDF3* and WT plants under control conditions. Fig. 6B shows the expression levels of the analysed genes in transgenic lines, where they exhibited higher values (from two- to fourfold) than in WT plants. These data indicated that SlCDF1 and SlCDF3 might be upstream activators in drought and salt stress pathways, acting directly or indirectly on the expression of different stress-regulated target genes.

Overexpression of SlCDF3 *in transgenic* Arabidopsis *plants induces metabolic changes and accumulation of specific compounds*

As drought and salt stress are known determinants that promote substantial physiological and metabolic rearrangements in plants (Rizhsky *et al.*, 2004; Seki *et al.*, 2007), we carried out non-targeted metabolite profiling to address the question of whether the ectopic expression of *SlCDF3* in *Arabidopsis* translates into a detectable alteration of the plant's metabolome. Principal component analysis (PCA) of the retention time, intensity, and accurate mass identity matrices, carried out to compare approximately 1000 molecular features per sample with each other, revealed that overexpression of *SlCDF3* resulted in a distinguishable alteration of the metabolome, as indicated by the clear clustering of the datasets (Fig. 8A). When we tried to identify the differentially abundant components causing the grouping in the PCA, we discovered that a great many of the differences were found among the group of

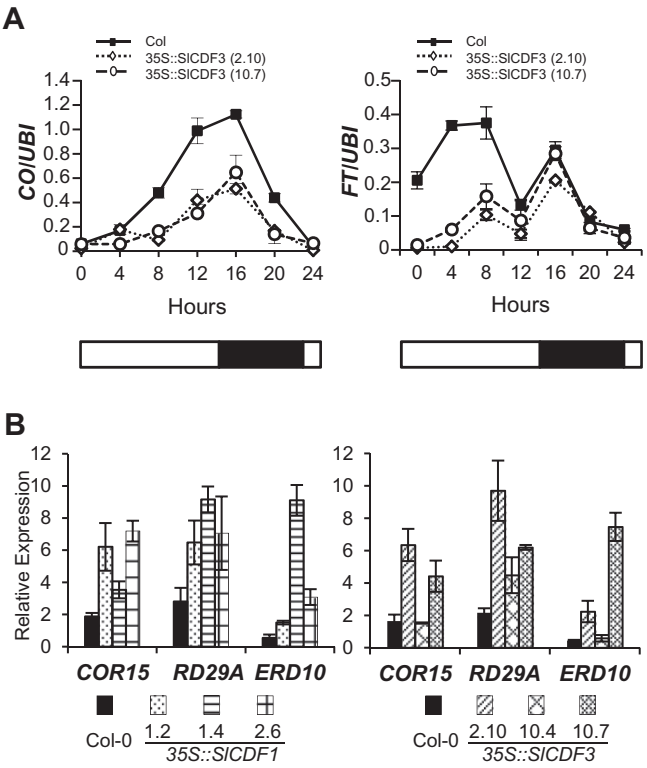

Fig. 6. Transcription analysis of flowering time and abiotic stress-responsive genes in *35S::SlCDF1* and *35S::SlCDF3* lines. (A) mRNA levels of *CO* and *FT* genes were analysed by qRT-PCR in *35S::SlCDF3* (L2.10 and L10.7) and control plants (Col-0). Total RNA was extracted from 10-d-old seedlings and harvested at the indicated times throughout a LD. White and black bars along the horizontal axis represent light and dark periods, respectively. (B) Expression of *COR15*, *RD29A*, and *ERD10* genes was analyzed by qRT-PCR on 3-week-old *35S::SlCDF1* (L1.2, L1.4, and L2.6), *35S::SlCDF3* (L2.10, L10.4, and L10.7) and control (Col-0) plants. Expression of the *Arabidopsis UBIQUITIN10* gene (Czechowski *et al.*, 2005) was used as reference gene. All data are expressed as means ±SE of three independent pools of extracts. Three technical replicates were performed for each extract.

small and polar compounds, containing for example sugars, amino acids, and small acids. As an example, the increased abundance of glutamine in the overexpressing lines compared with the WT is shown in Fig. 8B and C. Hence, we focused our analyses on these polar compounds and performed a targeted metabolomic profiling by gas chromatography-mass spectrometry to study the relative levels of different polar compounds, including proteinogenic amino acids as well as four other amino acids, eight distinct sugars plus two sugar alcohols, and eight small acids, extracted from 12-d-old WT and *35S::SlCDF3* (L2.10 and L10.7) transgenic plants, grown under non-stress conditions. As shown in Fig. 8D and Supplementary Table S4 (available at *JXB* online), comparison of gas chromatography profiles revealed a number of clear differences between the control and overexpressing lines. Overexpression of *SlCDF3* in *Arabidopsis* significantly induced the accumulation of sugars like sucrose (2.5-fold) and amino acids like γ-aminobutyric acid (GABA, 2-fold), L-proline (2.2-fold) and L-glutamine (1.8-fold), and succinate (1.3-fold), while the amount of malate and gluconate decrease by up to 24 and 34.9 %, respectively, relative to the control. Consistent with the expected similar effects in both *SlCDF3* overexpressing lines, most sugars appeared at comparable levels. Interestingly, these lines showed an important increase in sucrose compared with the WT. As glucose and fructose, the two monomeric building blocks of sucrose, showed no considerable reductions, it may be concluded that *SlCDF3* overexpression either causes a change in carbon partitioning favouring the production of sucrose over that of starch, or that CO_2 fixation rates are generally increased. Finally, overexpression of *SlCDF3* did not trigger the accumulation of organic acids, except succinate, as reflected by its increased concentration in both transgenic lines grown under control conditions (Fig. 8D).

Discussion

DOF proteins are plant-specific TFs that participate in different developmental and physiological processes (Lijavetzky *et al.*, 2003; Moreno-Risueno *et al.*, 2007*a*). In this work, we identified and characterized tomato *DOF* genes, homologous to *Arabidopsis CDF* genes, and found that the encoded proteins possessed transcriptional activation ability. Furthermore, we have provided evidence for their participation in the control of flowering time and abiotic stress responses.

SlCDFs share a high degree of sequence similarity but display different DNA-binding affinities and diverse transcriptional activation capabilities

We searched the complete tomato genome sequence and identified 34 genes encoding DOF proteins. In accordance with previous studies in *Arabidopsis* (Lijavetzky *et al.*, 2003), these 34 genes were divided into four groups (A–D) on the basis of similarities in their DNA-binding domains. Within group D, we found five tomato genes with high levels of sequence similarity to *Arabidopsis* CDFs. The encoded proteins showed conservation not only in their DNA-binding domain but also in their C-terminal region, which contained three conserved motifs of 21, 22, and 33 aa, respectively, which were reported to be essential for the protein–protein interaction with the C-terminal kelch repeat domain of the F-box proteins FKF1 and LKP2 (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). In addition, these three motifs are also conserved in homologous proteins from other species, such as *Jatropha curcas* (JcDOF3; Yang *et al.*, 2011), *Brachypodium distachyon* (BdDOF4, -11, -16, -20, and -22; Hernando-Amado *et al.*, 2012), and *Solanum tuberosum* (StCDF1; Kloosterman *et al.*, 2013). Interestingly, two allelic variants of potato *StCDF1* (*StCDF1.2* and *StCDF1.3*) lacking the C-terminal end have been reported to be impaired in their interaction with the FKF1–GI complex. As a consequence, this results in major defects in plant maturity and tuber development (Kloosterman *et al.*, 2013). Consistent with these data, it may be concluded that the three identified

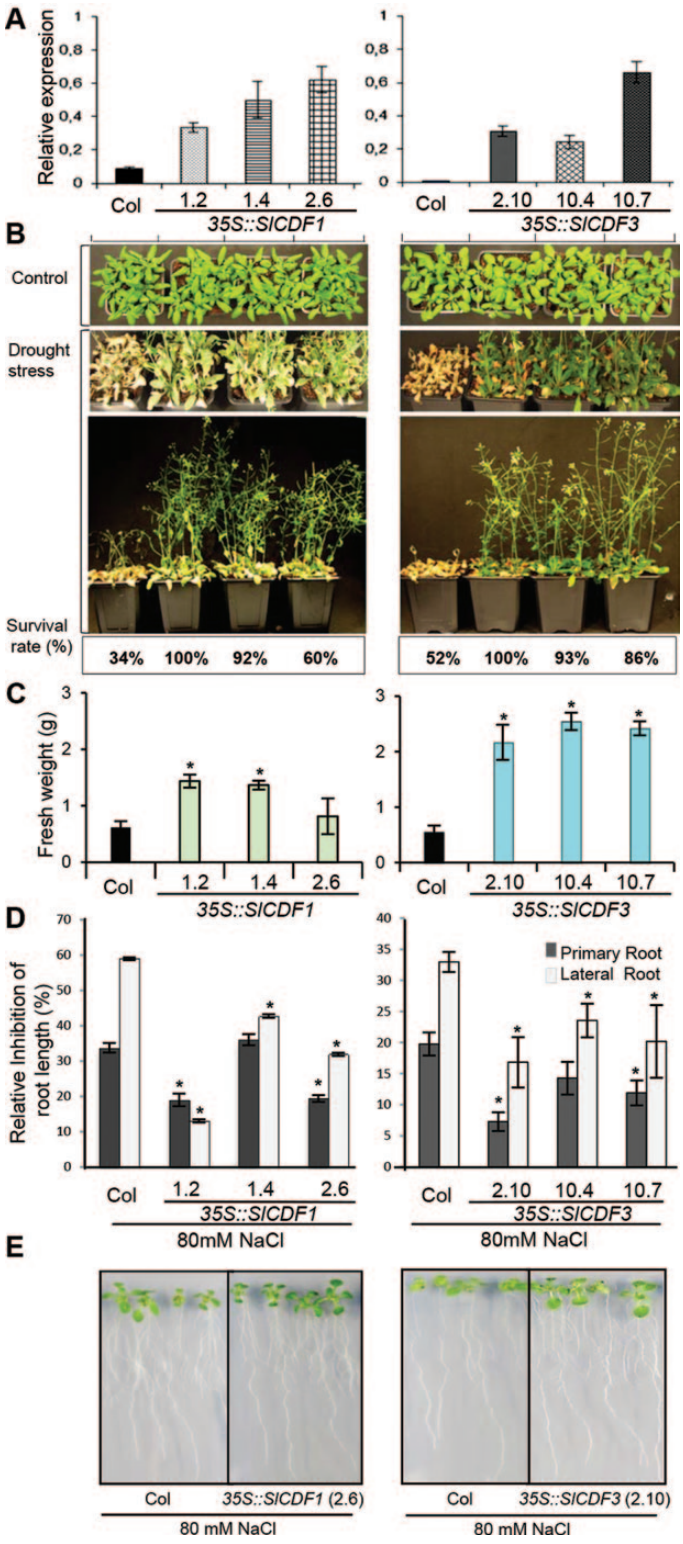

Fig. 7. Drought and salt stress tolerance of *35S::SlCDF1* and *35S::SlCDF3* plants. (A) Transcription analysis of tomato *SlCDF1* and *SlCDF*3 genes in different T3 independent *35S::SlCDF1* (L.12, L1.4, and L2.6) and *35S::SlCDF3* (L2.10, L10.4, and L10.7) transgenic lines. *SlCDF1-3* expression was analysed by qRT-PCR in *Arabidopsis* plants. Expression of the *Arabidopsis UBIQUITIN10* gene (Czechowski *et al.*, 2005) was used as a reference gene. Data are expressed as means ±SE of three independent extractions. Three technical replicates were performed for each extraction. (B) Drought stress tolerance was estimated by scoring fresh weight and survival rates of 2-week-old *35S::SlCDF1* (L1.2, L1.4, and L2.6), *35S::SlCDF3* (L2.10, L10.4, and L10.7), and control (Col-0) plants, which were maintained for 15 d without irrigation and then given 10 d of rewatering. Representative images are shown of plants before and after the treatment. Survival rates are indicated under the photographs. (C) Fresh weight data are expressed as means ±SE of three independent experiments with five plants each. Asterisks indicate significant differences between Col-0 and *35S::SlCDF1*- or *35S::SlCDF3*-overexpressing lines ($P<0.01$; ANOVA, followed by a Student–Newman–Keuls test). (D) Salt stress tolerance estimated by determining the reduction of primary and lateral growth of *35S::SlCDF1* (L1.2, L1.4, and L2.6), *35S::SlCDF3* (L2.10, L10.4, and L10.7), and control (Col-0) plants after 10 d in MS medium supplemented with 80 mM NaCl and represented as the percentage reduction relative to standard conditions. Data are expressed as means ±SE of three independent experiments with at least 20 plants each. Asterisks indicate significant differences between Col-0 and *35S::SlCDF1*- or *35S::SlCDF3*-overexpressing lines ($P<0.01$; ANOVA, followed by a Student–Newman–Keuls test). (E) Representative images of Col-0, *35S::SlCDF1* (L2.6), and *35S::SlCDF3* (L2.10) plants after the treatments.

C-terminal motifs are common features of CDF proteins, through which the regulatory mechanisms controlled by CDFs are determined.

Subcellular localization and yeast one-hybrid assays conducted in this study showed that the identified tomato SlCDFs are nuclear factors that bind to the core 5′-TAAAG-3′ DOF *cis*-DNA element (Yanagisawa and Schmidt, 1999) with different binding affinities. Transactivation assays confirmed these results and indicated that SlCDFs can act as transcriptional activators, again to different extents. While SlCDF1 and SlCDF2 exhibited only low-level transcriptional activation capabilities, SlCDF3, SlCDF4, and SlCDF5 displayed higher transcriptional activation capacity. Consistent with these data, overexpression of *SlCDF1* and *SlCDF3* in *Arabidopsis* promotes the expression of *COR15*, *RD29A*, and *ERD10*. Whether they act directly or indirectly as upstream activators remains to be elucidated. In contrast, we found that the overexpression of *SlCDF3* resulted in reduced expression of both *CO* and *FT* genes, most likely acting as a target repressor, as reported for the *Arabidopsis* CDF1 protein (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). It should be noted that the DOF domain was first identified as a DNA-binding domain but was also reported as a bifunctional domain for DNA binding and protein–protein interactions (Mackay and Crossley, 1998). Differences in the activities of DOF TFs have been associated with the core DOF domain (Yanagisawa, 2004) as well as their protein–protein interactions with other TFs. In fact, the DOF domain participates in the interaction with other classes of TFs like bZIP proteins or HMG proteins, which in turn modify their transcription capabilities (Zhang *et al.*, 1995; Vicente-Carbajosa *et al.*, 1997; Yanagisawa, 1997; Krohn *et al.*, 2002). For example, the *Arabidopsis* DOF protein

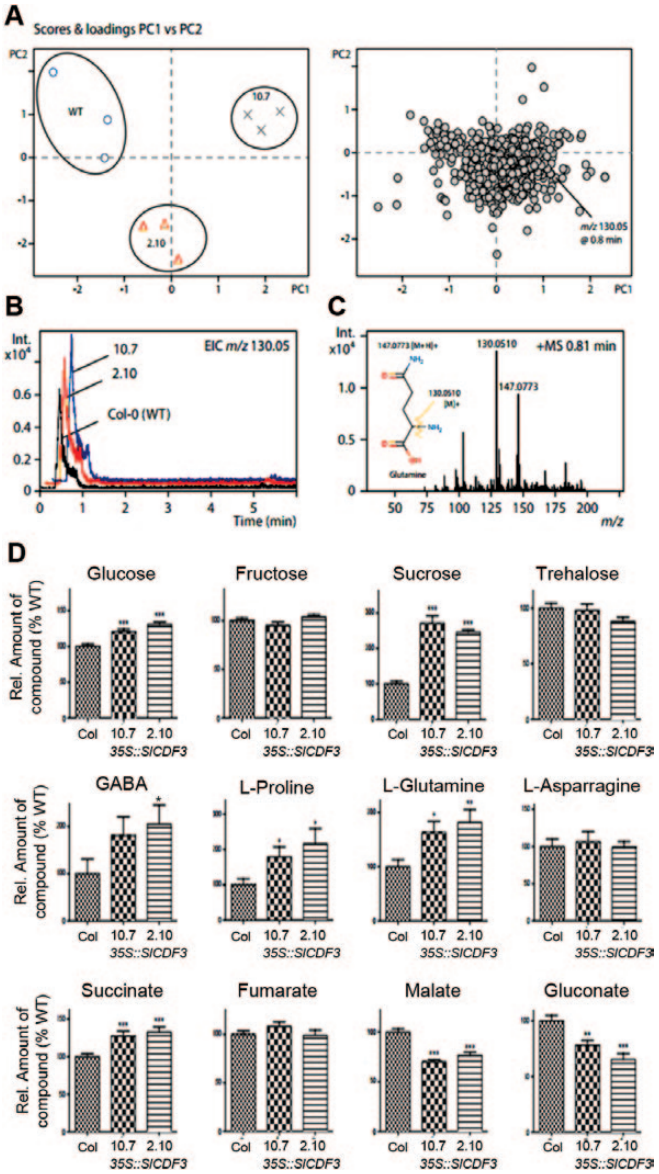

Fig. 8. Metabolic analyses of *35S::SlCDF3* and WT plants. (A) PCA of recorded, non-targeted metabolic profiles using Profile Analysis (Bruker Daltonics, Bremen, Germany). Projection plots are shown for principal component 1 (PC1, 19% variance explained) and PC2 (15%). Distinct grouping supports the different genotypes analysed: WT control samples or overexpression lines 2.10 and 10.7, respectively. (B) Extracted ion chromatograms (EICs) for mass *m/z* 130.05 at 0.81 min reveal induction of the compound in the overexpression lines. (C) The accurate mass of the parent ion and its isotopic pattern led to the identification of L-glutamine. (D) Relative quantities (% of WT) of selected metabolites analysed by Gas chromatography-selected ion monitoring-mass spectrometry. Results are shown as means ±SE (n=15). Similar results were obtained in five independent experiments. (Student's t-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (This figure is available in colour at *JXB* online.)

OBP1 was identified as a protein interacting with bZIP proteins OBF4 and OBF5 associated with stress responses (Zhang *et al.*, 1995). Altogether, these data suggest that the identified SlCDFs could display different transcription activities depending on target gene promoters and the combinatorial interactions with other TFs present in a particular tissue or under different environmental conditions.

Expression of SlCDFs follows a circadian rhythm with two different patterns

Diurnal oscillation of transcript levels of *CDF*s has been reported for *Arabidopsis* and other species under day/night and constant light conditions (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009; Iwamoto *et al.*, 2009; Yang *et al.*, 2011). *AtCDF*s exhibit different diurnal expression patterns that can be classified in two different groups: *CDF1*, *CDF2*, *CDF3*, and *CDF5* show maximum expression at the beginning of the light period, decreasing progressively thereafter to a minimum between 16 and 20 h, then rising again during dawn; and the group comprising *CDF4*, whose transcript levels rise progressively from dawn and decrease at the end of the night (Fornara *et al.*, 2009). In the present study, the identified tomato *SlCDF*s exhibit similar diurnal expression patterns under LD and LL conditions, supporting the assumption that they are true homologues of the *Arabidopsis* CDFs. Interestingly, their gene expression patterns could be also classified in two groups: the group of *SlCDF1* and *SlCDF3* exhibit a maximum at the beginning of the day, while *SlCDF2*, *SlCDF4*, and *SlCDF5* exhibit maximum levels during the night period, suggesting that the family of CDFs might display different function (at least two conserved functions) and regulate specific target genes at different periods of the day.

Expression of tomato SlCDF *genes in* Arabidopsis *unveils a conserved function in the control of flowering time*

It is well established that regulation of temporal expression of the transcription factor CO is crucial to control the photoperiodic flowering in *Arabidopsis* and other photoperiod-sensitive species (Suárez-López *et al.*, 2001; Mizoguchi *et al.*, 2005). The induction of *CO* mRNA by light under LD conditions, but not under short-day conditions, is a key element for the triggering of flowering, as light treatment is necessary for the stabilization of CO protein (Valverde *et al.*, 2004; Jang *et al.*, 2008) and the subsequent activation of *FT* transcription (Takada and Goto, 2003; An *et al.*, 2004; Wigge *et al.*, 2005; Yoo *et al.*, 2005). In addition, the *Arabidopsis* CDFs act redundantly in repressing *CO* transcription to modulate the diurnal expression rhythm (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). Our results showed that the overexpression of tomato *SlCDF3*, in analogy to *Arabidopsis CDF1*, promoted late flowering in *Arabidopsis*. Interestingly, *SlCDF3* overexpression also led to a reduction in the mRNA levels of *CO* and *FT*, the natural direct targets of the *Arabidopsis* counterpart (Fig. 6), which is in support of a conserved functionality. Nevertheless, it should be noted that tomato plants are photoperiod insensitive in their native habitats and there is no single environmental factor known to be critical for flower induction in this species (Heuvelink and Dorais, 2005). Several factors such as light intensity, temperature, and number of leaves

affect the time of flowering in tomato (Calvert, 1959; Hussey, 1963; Kinet, 1977; Uzun, 2006), a process considered to be controlled by intraplant competition for assimilates (Sachs and Hackett, 1969; Atherton and Harris, 1986; Dieleman and Heuvelink, 1992). Notably, key regulatory genes like *CO* and the *CDF*s implicated in the photoperiodic flowering pathway are also present in tomato (Pnueli *et al.*, 1998, 2001; Carmel-Goren *et al.*, 2003; Ben-Naim *et al.*, 2006). Our results suggest that some of the identified tomato SlCDFs, like SlCDF3, might retain some functions in the control of flowering time through similar molecular mechanisms to those observed when expressed in *Arabidopsis*, but also that they might have additional functions in tomato.

SlCDFs involvement in abiotic stress responses

As revealed by qRT-PCR expression analyses, all *SlCDF*s responded to different abiotic stresses like salt, drought, and extreme temperatures with different timing and spatial expression patterns in roots and shoots, suggesting that they might participate in abiotic stress responses. This observation led us to the generation and analyses of *35S::SlCDF1* and *35S::SlCDF3* transgenic *Arabidopsis* plants. We could confirm that the overexpression of *SlCDF1* and *SlCDF3* resulted in increased tolerance to both salt and drought stress, as shown by survival rates and root length assays. Moreover, both overexpressing lines exhibited higher expression levels of abiotic stress-responsive genes, like *COR15*, *RD29A*, and *ERD10*, under non-stress conditions, which indicated that SlCDFs might function as upstream regulators in drought and salt stress response pathways**.** Metabolic profiling of *35S::SlCDF3* plants showed increased levels of proline, glutamine, GABA, and sucrose. These compounds are normally accumulated under water stress and salinity conditions (Hoekstra *et al.*, 2001; Rizhsky *et al.*, 2004), aiding stress tolerance through osmotic adjustment, detoxification of ROS, and intracellular pH regulation (Rajasekaran *et al.*, 2000; Claussen, 2005; Munns and Tester, 2008; Bressan *et al.*, 2009; Chaves *et al.*, 2009). Their significantly increased levels, promoted by the overexpression of *SlCDF3* in *Arabidopsis*, seemingly contribute to improved drought and salt tolerance, as their content has been correlated with stress tolerance (Kerepesi and Galiba, 2000; Farrant and Moore, 2011; Pinheiro and Chaves, 2011). Altogether, our results strongly support the participation of SlCDFs in plant responses and tolerance to abiotic stress conditions.

Impact of SlCDF *expression on carbon/nitrogen metabolism*

The *SlCDF*s exhibited different expression patterns during development. However, with the exception of *SlCDF3*, all were expressed during vegetative development at high levels, especially in young tissues like cotyledons. In organs with contrasting sink and source activities like mature vegetative tissues of shoots and roots, and reproductive tissues, such as flowers and fruits, they are also differentially expressed. This may highlight precise tissue-specific functions for the SlCDFs in controlling the expression levels of particular subsets of genes and consequently specific metabolic processes. In this regard, the metabolic analyses of *35S::SlCDF3* plants showed that the overexpression of *SlCDF3* transcription factor in *Arabidopsis* resulted in significant metabolic alterations. Specifically, we observed higher levels of sucrose and of certain amino acids, indicative of increased nitrogen assimilation, as reported previously for other DOF TFs (Yanagisawa *et al.*, 2004). In this line, our studies also revealed a higher content of succinate and GABA. The hypothesis that GABA acts as a temporary nitrogen storage pool could explain the increased concentration of this non-proteinogenic amino acid (Beuve *et al.*, 2004). On the other hand, upregulation of the pathway that converts glutamate to succinate via GABA would explain the rise in succinate content (Rhodes *et al.*, 1999). Glutamic acid metabolism via the GABA shunt could be of considerable importance in the nitrogen economy of plants (Shelp *et al.*, 1999, 2006). As carbon and nitrogen metabolites mutually influence each other in a fine balance between carbon and nitrogen metabolism (Yanagisawa *et al.*, 2004; Kurai *et al.*, 2011), the higher content of sucrose in *35S::SlCDF3* transgenic plants suggests that CO_2 fixation could be also stimulated to maintain the carbon/nitrogen balance. Hence, we hypothesize that *SlCDF* genes could be involved in the regulation of primary metabolism in different tissues and under precise developmental and stress conditions.

CDFs at the interplay between environmental conditions and flowering time

The results of our study confirmed a previously reported and salient feature of CDFs in the control of flowering time. Specifically, the overexpression of AtCDFs in phloem companion cells leads to a delay in flowering under LD conditions, although with a different impact in *Arabidopsis* (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). Here, we could demonstrate conservation of this function for specific tomato CDFs, which are able to reproduce the same phenotype when expressed in *Arabidopsis*. Flowering time is critical in the plant life cycle, yet plants must closely monitor the environmental state to determine the onset of flowering for reproductive success. Intriguingly, the data presented here revealed that, besides the participation of some *SlCDF* genes in the control of flowering in photoperiod-sensitive species, they also display additional functions. Notably, SlCDFs regulate the expression of genes involved in abiotic stress responses. Moreover, metabolic analyses of *SlCDF*-overexpressing plants showed accumulation of precise compounds that mitigate abiotic stress conditions. They also showed important changes in particular metabolites, like increased levels of sucrose and certain amino acids, typically associated with physiological states like the nutrient salvage and recycling under senescence programmes (Jones, 2013) or the mobilization and relocation of resources from source to sink organs. This information opens up the possibility of further investigating the links of CDF function in the adaptation to environmental conditions and the progression from vegetative to reproductive phases.

Additional research and in-depth physiological characterization of transgenic plants for the different *SlCDF* genes, currently underway, will clarify the precise role of these genes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Phylogenetic tree of *Arabidopsis* and tomato DOF proteins.

Supplementary Table S1. *S. lycopersicum* DOF protein sequences.

Supplementary Table S2. Gene structures of encoded *S. lycopersicum* DOF transcription factors.

Supplementary Table S3. Primers designed for real-time PCR, expected size, and concentration used.

Supplementary Table S4. Metabolite analyses of WT and *35S::SlCDF3* plants.

Supplementary File 1. Methods for metabolite analyses.

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References

Abràmoff MD, Magalhães PJ, Ram SJ. 2004. Image Processing with ImageJ. *Biophotonics International* **11,** 36–42.

Abuqamar S, Luo H, Laluk K, Mickelbart MV, Mengiste T. 2009. Crosstalk between biotic and abiotic stress responses in tomato is mediated by the AIM1 transcription factor. *The Plant Journal* **58,** 347–360.

Alonso R, Oñate-Sánchez L, Weltmeier F, Ehlert A, Diaz I, Dietrich K, Vicente-Carbajosa J, Dröge-Laser W. 2009. A pivotal role of the basic leucine zipper transcription factor bZIP53 in the regulation of Arabidopsis seed maturation gene expression based on heterodimerization and protein complex formation. *Plant Cell* **21,** 1747–1761.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhan Z, Miller W, Lipman, DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

An H, Roussot C, Suarez-Lopez P, Corbesier L, Vincent C, Pineiro M, Hepworth S, Mouradov A, Justin S, Turnbull C, Coupland G. 2004. CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. *Development* **131,** 3615–3626.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annual Review of Plant Physiology* **55,** 373–399.

Artimo P, Jonnalagedda M, Arnold K, *et al.* 2012. ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research* **40,** 1–7.

Atherton JG, Harris GP. 1986. Flowering. In: Atherton JG, Rudic J. eds. *The Tomato Crop: A Scientific Basis for Improvement*. New York: Chapman and Hall, 167–200.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME Suite: tools for motif discovery and searching. *Nucleic Acids Research* **37,** 202–208.

Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, Samach A, Lifschitz E. 2006. The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *The Plant Journal* **46,** 462–476.

Beuve N, Rispail N, Laine P, Cliquet J-B, Ourry A, Le Deunff E. 2004. Putative role of γ-aminobutyric acid (GABA) as a long distance signal in up-regulation of nitrate uptake in *Brassica napus* L. *Plant, Cell & Environment* **27,** 1035–1046.

Blumwald E. 2000. Sodium transport and salt tolerance in plants. *Current Opinion in Cell Biology* , **12,** 431–434.

Bombarely A, Menda N, Tecle IY, Buels RM, Strickler S, Fischer-York T, Pujar A, Leto J, Gosselin J, Mueller LA. 2011. The Sol Genomics Network (solgenomics.net): growing tomatoes using Perl. *Nucleic Acids Research* **39,** 1149–1155.

Bressan R, Bohnert H, Zhu J-K. 2009. Abiotic stress tolerance: from gene discovery in model organisms to crop improvement. *Molecular Plant* **2,** 1–2.

Calvert A. 1959. Effect of the early environment on the development of flowering in tomato: II. Light and temperature interactions. *Journal of Horticultural Science* **34,** 154–162.

Carmel-Goren L, Liu YS, Lifschitz E, Zamir D. 2003. The SELF-PRUNING gene family in tomato. *Plant Molecular Biology* **52,** 1215–1222.

Chaves MM, Flexas J, Pinheiro C. 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* **103,** 551–560.

Claussen W. 2005. Proline as a measure of stress in tomato plants. *Plant Science* **168,** 241–248.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Cuartero J, Fernández-Muñoz R, González-Fernández JJ. 1995. Estreses abióticos. In: Nuez F, ed. *El cultivo del tomate*. Mundi Prensa, Madrid, 352–383.

Cuartero J, Fernández-Muñoz R. 1999. Tomato and salinity. *Scientia Horticulturae* **78,** 83–125.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization. *Plant Physiology* **139,** 5–17.

Diaz I, Vicente-Carbajosa J, Abraham Z, Martínez M, Isabel-La Moneda I, Carbonero P. 2002. The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates

endosperm-specific genes during seed development. *The Plant Journal* **29,** 453–64.

Dieleman JA, Heuvelink E. 1992. Factors affecting the number of leaves preceding the first inflorescence in the tomato. *Journal of Horticultural Science* **67,** 1–10.

Farrant JM, Moore JP. 2011. Programming desiccation-tolerance: from plants to seeds to resurrection plants. *Current Opinion in Plant Biology* **14,** 340–345.

Fiehn O, Kopka J, Trethewey RN, Willmitzer L. 2000. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Analytical Chemistry* , 3573–3580.

Fornara F, Panigrahi KCS, Gissot L, Sauerbrunn N, Rühl M, Jarillo JA, Coupland G. 2009. Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. *Developmental Cell* **17,** 75–86.

Gaquerel E, Heiling S, Schoettner M, Baldwin IT. 2010. Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. *Journal of Agricultural and Food Chemistry* **58,** 9418–9427.

Gardiner J, Sherr I, Scarpella E. 2010. Expression of DOF genes identifies early stages of vascular development in Arabidopsis leaves. *International Journal of Developmental Biology* **54,** 1389–1396.

Gong P, Zhang J, Li H, *et al.* 2010. Transcriptional profiles of drought-responsive genes in modulating transcription signal transduction, and biochemical pathways in tomato. *Journal of Experimental Botany* **61,** 3563–3575.

Goodstein DM, Shu S, Howson R, *et al.* 2012. Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Research* **40,** 1178–1186.

Gualberti G, Papi M, Bellucci L, Ricci I, Bouchez D, Camilleri C, Costantino P, Vittorioso P. 2002. Mutations in the Dof Zinc finger genes DAG2 and DAG1 influence with opposite effects the germination of Arabidopsis seeds. *Plant Cell* **14,** 1253–1263.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52,** 696–704.

Gullberg J, Jonsson P, Nordstrom A, Sjostrom M, Moritz T. 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. *Analytical Biochemistry* **331,** 283–295.

Guo Y, Qin G, Gu H, Qu L-J. 2009. Dof5.6/HCA2, a Dof transcription factor gene, regulates interfascicular cambium formation and vascular tissue development in Arabidopsis. *The Plant Cell* **21,** 3518–3534.

Haupt-Herting S, Klug K, Fock HP. 2001. A new approach to measure gross CO_2 fluxes in leaves. Gross CO_2 assimilation, photorespiration, and mitochondrial respiration in the light in tomato under drought stress. *Plant Physiology* **126,** 388–396.

Hernando-Amado S, González-Calle V, Carbonero P, Barrero-Sicilia C. 2012. The family of DOF transcription factors in *Brachypodium distachyon*: phylogenetic comparison with rice and barley DOFs and expression profiling. *BMC Plant Biology* **12**, 202.

Heuvelink E, Dorais M. 2005. Crop growth and Yield. In: Heuveling E, ed. *Tomatoes*. *Crop production*. Science in Horticulture. CABI Publishing, Wallingford, UK, 85–144.

Hoagland DR, Arnon D. 1950. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* **347,** 1–32.

Hoekstra FA, Golovina EA, Buitink J. 2001. Mechanisms of plant desiccation tolerance. *Trends in Plant Science* **6,** 431–438.

Hoffman N, Ko K, Milkowski D, Pichersky E. 1991. Isolation and characterization of tomato cDNA and genomic clones encoding the ubiquitin gene ubi3. *Plant Molecular Biology* **17,** 1189–1201.

Huang Z, Zhang Z, Zhang X, Zhang H, Huang D, Huang R. 2004. Tomato TERF1 modulates ethylene response and enhances osmotic stress tolerance by activating expression of downstream genes. *FEBS Letters* **27,** 110–116.

Hussey G. 1963. Growth and development in young tomato. The effect of temperature and light intensity on growth of the shoot apex and leaf primordial. *Journal of Experimental Botany* **14,** 316–325.

Imaizumi T, Schultz TF, Harmon FG, Ho L, Kay S. 2005. FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. *Science* **309,** 293–297.

Iwamoto M, Higo K, Takano M. 2009. Circadian clock- and phytochrome-regulated Dof-like gene, Rdd1, is associated with grain size in rice. *Plant, Cell & Environment* **32,** 592–603.

Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, Valverde F, Coupland G. 2008. Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *EMBO Journal* **27,** 1277–1288.

Jones ML. 2013. Mineral nutrient remobilization during corolla senescence in ethylene-sensitive and -insensitive flowers. *AoB PLANTS* **5,** plt023.

Karimi M, Depicker A, Hilson P. 2007. Recombinational cloning with plant Gateway vectors. *Plant Physiology* **145,** 1144–1154.

Kerepesi I, Galiba G. 2000. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Science* **40,** 482–487.

Kinet JM. 1977. Effect of light conditions on the development of the inflorescence in tomato. *Scientia Horticulturae* **6,** 15–26.

Kirby J, Kavanagh TA. 2002. NAN fusions: a synthetic sialidase reporter gene as a sensitive and versatile partner for GUS. *Plant Journal* **32,** 391–400.

Kloosterman B, Abelenda J A, Gomez M, *et al.* 2013. Naturally occurring allele diversity allows potato cultivation in northern latitudes. *Nature* **495,** 246–250.

Konishi M, Yanagisawa S. 2007. Sequential activation of two Dof transcription factor gene promoters during vascular development in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* **45,** 623–629.

Krohn NM, Yanagisawa S, Grasser KD. 2002. Specificity of the stimulatory interaction between chromosomal HMGB proteins and the transcription factor Dof2 and its negative regulation by protein kinase

CK2-mediated phosphorylation. *Journal of Biological Chemistry* **277,** 32438–32444.

Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R. 2011. Introduction of the *ZmDof1* gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. *Plant Biotechnology Journal* **9,** 826–837.

Kushwaha H, Gupta S, Singh V, Rastogi S, Yadav D. 2011. Genome wide identification of Dof transcription factor gene family in sorghum and its comparative phylogenetic analysis with rice and Arabidopsis. *Molecular Biology Reports* **38,** 5037–5053.

Lakhssassi N, Doblas VG, Rosado A, Valle AE, Posé D, Jimenez AJ, Castillo AG, Valpuesta V, Borsani O, Botella MA. 2012. The Arabidopsis TETRATRICOPEPTIDE THIOREDOXIN-LIKE gene family is required for osmotic stress tolerance and male sporogenesis. *Plant Physiology* **158,** 1252–1266.

Lee HE, Shin D, Park SR, *et al.* 2007 Ethylene responsive element binding protein 1 (StEREBP1) from *Solanum tuberosum* increases tolerance to abiotic stress in transgenic potato plants. *Biochemical and Biophysical Research Communications* **353,** 863–868.

Lijavetzky D, Carbonero P, Vicente-Carbajosa J. 2003. Genome-wide comparative phylogenetic analysis of the rice and Arabidopsis Dof gene families. *BMC Evolutionary Biology* **3,** 17.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-Time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25,** 402–408.

Mackay JP, Crossley M. 1998. Zinc fingers are sticking together. *Trends in Biochemical Sciences* **23,** 1–4.

Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P. 1998. An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. *The Plant Journal* **16,** 53–62.

Mizoguchi T, Wright L, Fujiwara S, *et al.* 2005. Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *Plant Cell* **17,** 2255–2270.

Moreno-Risueno M, Martínez M, Vicente-Carbajosa J, Carbonero P. 2007a. The family of DOF transcription factors: from green unicellular algae to vascular plants. *Molecular Genetics and Genomics* **277,** 379–390.

Moreno-Risueno MA, Díaz I, Carrillo L, Fuentes R, Carbonero P. 2007b. The HvDOF19 transcription factor mediates the abscisic acid- dependent repression of hydrolase genes in germinating barley aleurone. *The Plant Journal* **51,** 352–365.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Physiology* **59,** 651–681.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of Bioscience and Bioengineering* **104,** 34–41.

Nuez F, Prohens J. 2008. Fabaceae, Liliaceae, Solanaceae, and Umbelliferae, Vegetables II. In: Nuez, F, Prohens J, eds. *Handbook of plant breeding*. Springer, Berlin, 249–327.

Oñate-Sánchez L, Vicente-Carbajosa J. 2008. DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Research Notes*. **1,** 93.

Orellana S, Yañez M, Espinoza A, Verdugo I, González E, Ruiz-Lara S, Casaretto JA. 2010. The transcription factor SlAREB1 confers drought, salt stress tolerance and regulates biotic and abiotic stress-related genes in tomato. *Plant, Cell & Environment* **33,** 2191–2208.

Papi M, Sabatini S, Altamura MM, Hennig L, Schäfer E, Costantino P, Vittorioso P. 2002. Inactivation of the phloem-specific Dof zinc finger gene DAG1 affects response to light and integrity of the testa of Arabidopsis seeds. *Plant Physiology* **128,** 411–417.

Papi M, Sabatini S, Bouchez D, Camilleri C, Costantino P, Vittorioso P. 2000. Identification and disruption of an Arabidopsis zinc finger gene controlling seed germination. *Genes & Development* **14,** 28–33.

Pinheiro C, Chaves MM. 2011. Photosynthesis and drought: can we make metabolic connections from available data? *Journal of Experimental Botany* **62,** 869–882.

Pnueli L, Carmel-Goren L, Hareven D, Gutfinger T, Alvarez J, Ganal M, Zamir D, Lifschitz E. 1998. The SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of CEN and TFL1. *Development* **125,** 1979–1989.

Pnueli L, Gutfinger T, Hareven D, Ben-Naim O, Ron N, Adir N, Lifschitz E. 2001. Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. *Plant Cell* **13,** 2687–2702.

Rajasekaran LR, Aspinall D, Pale, LG. 2000. Physiological mechanism of tolerance of *Lycopersicon* spp. exposed to salt stress. *Canadian Journal of Plant Science* **80,** 151–159.

Rhodes D, Verslues PE, Sharp RE. 1999. Role of amino acids. In: Singh BK. ed. *Abiotic stress resistance. Plant amino acids: biochemistry and biotechnology*. Marcel Dekker, New York, 319–356.

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. 2004. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. *Plant Physiology* **134,** 1683–1696.

Rueda-López M, Crespillo R, Cánovas FM, Avila C. 2008. Differential regulation of two glutamine synthetase genes by a single Dof transcription factor. *The Plant Journal* **56,** 73–85.

Sachs RM, Hackett WP. 1969. Control of vegetative and reproductive development in seed plants. *HortScience* **4,** 103–107.

Sawa M, Nusinow DA, Kay SA, Imaizumi T. 2007. FKF1 and GIGANTEA Complex formation is required for day-length measurement in Arabidopsis. *Science* **318,** 261–265.

Seki M, Umezawa T, Urano K, Shinizaki K. 2007. Regulatory metabolic networks in drought stress responses. *Current Opinion in Plant Biology* **10,** 296–302.

Seong ES, Kwon S, Ghimire BK, *et al.* 2008. LebZIP2 induced by salt and drought stress and transient overexpression by

Agrobacterium. *Biochemistry and Molecular Biology Reports* **41,** 693–698.

Shannon MC, Grieve CM. 1999. Tolerance of vegetable crops to salinity. *Scientia Horticulturae* **78,** 5–38.

Shaw LM, McIntyre CL, Gresshoff PM, Xue G-P. 2009. Members of the Dof transcription factor family in *Triticum aestivum* are associated with light-mediated gene regulation. *Functional & Integrative Genomics* **9,** 485–498.

Shelp BJ, Bown AW, Faure D. 2006. Extracellular γ-aminobutyrate mediates communication between plants and other organisms. *Plant Physiology* **142,** 1350–1352.

Shelp BJ, Bown AW, McLean MD. 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends in Plant Science* **4,** 446–452.

Skirycz A, Jozefczuk S, Stobiecki M, Muth D, Zanor MI, Witt I, Mueller-Roeber B. 2007. Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*. *New Phytologist* **175,** 425–438.

Skirycz A, Reichelt M, Burow M, *et al.* 2006. DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis. *The Plant Journal* **47,** 10–24.

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland, G. 2001. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature* **410,** 1116–1120.

Sun S-J, Guo S-Q, Yan, X, Bao Y-M, Tang H-J, Sun H, Huang J, Zhang H-S. 2010. Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice. *Journal of Experimental Botany* **61,** 2807–2818.

Takada S, Goto K. 2003. TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by *CONSTANS* in the vascular tissues of leaves to regulate flowering time. *Plant Cell* **15,** 2856–2865.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28,** 2731–2739.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25,** 4876–4882.

Uzun S. 2006. The quantitative effects of temperature and light on the number of leaves preceding the first fruiting inflorescence on the stem of tomato (*Lycopersicon esculentum*, Mill.) and aubergine (*Solanum melongena* L.). *Scientia Horticulturae* **109,** 142–146.

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. 2004. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303,** 1003–1006.

Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ. 1997. A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proceedings of the National Academy of Sciences, USA* **94,** 7685–7690.

Wang HW, Zhang B, Hao YJ, Huang J, Tian AG, Liao Y, Zhang JS, Chen, SY. 2007. The soybean Dof-type transcription factor genes, GmDof4 and GmDof11, enhance lipid content in the seeds of transgenic Arabidopsis plants. *The Plant Journal* **52,** 716–729.

Washio K. 2001. Identification of Dof proteins with implication in the gibberellin-regulated expression of a peptidase gene following the germination of rice grains. *Biochimica et Biophysica Acta* **1520,** 54–62.

Wei X, Chen M, Xiao J, Liu Y, Yu L, Zhang H, Wang, Y. 2010. Composition and bioactivity of tea flower polysaccharides obtained by different methods. *Carbohydrate Polymers* **79,** 418–422.

Weltmeier F, Ehlert A, Mayer CS, Dietrich K, Wang X, Schütze K, Alonso R, Harter K, Vicente-Carbajosa J, Dröge-Laser W. 2006. Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. *EMBO Journal* **25,** 3133–3143.

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D. 2005. Integration of spatial and temporal information during floral induction in Arabidopsis. *Science* **309,** 1056–1059.

Wu L, Chen X, Ren H, Zhang Z, Zhang H, Wang J, Wang XC, Huang R. 2007. ERF protein JERF1 that transcriptionally modulates the expression of abscisic acid biosynthesis-related gene enhances the tolerance under salinity and cold in tobacco. *Planta* **226,** 815–825.

Yamamoto MP, Onodera Y, Touno SM, Takaiwa, F. 2006. Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. *Plant Physiology* **141,** 1694–1707.

Yanagisawa S. 1997. Dof DNA-binding domains of plant transcription factors contribute to multiple protein–protein interactions. *European Journal of Biochemistry* **250,** 403–410.

Yanagisawa S. 2001. The transcriptional activation domain of the plant-specific Dof1 factor functions in plant, animal, and yeast cells. *Plant and Cell Physiology* **42,** 813–822.

Yanagisawa S. 2002. The Dof family of plant transcription factors. *Trends in Plant Science* **7,** 555–560.

Yanagisawa S. 2004. Dof domain proteins: plant-specific transcription factors associated with diverse phenomena unique to plants. *Plant and Cell Physiology* **45,** 386–391.

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T. 2004. Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. *Proceedings of the National Academy of Sciences, USA* **101,** 7833–7838.

Yanagisawa S, Schmidt RJ. 1999. Diversity and similarity among recognition sequences of Dof transcription factors. *The Plant Journal* **17,** 209–214.

Yanagisawa S, Sheen J. 1998. Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *Plant Cell* **10,** 75–89.

Yáñez M, Cáceres S, Orellana S, Bastías A, Verdugo I, Ruiz-Lara S, Casaretto J. 2009. An abiotic stress-responsive bZIP transcription factor from wild and cultivated tomatoes regulates stress-related genes. *Plant Cell Reports* **28,** 1497–1507.

Yang J, Yang MF, Zhan, WP, Chen F, Shen SH. 2011. A putative flowering-time-related Dof transcription factor gene, JcDof3, is

controlled by the circadian clock in *Jatropha curcas*. *Plant Science* **181,** 667–674.

Yoo SK, Chung KS, Kim J, Lee JH, Hong SM, Yoo SJ, Yoo SY, Lee JS, Ahn JH. 2005. *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS* T to promote flowering in Arabidopsis. *Plant Physiology* **139,** 770–778.

Zhang B, Chen W, Foley RC, Büttner M, Singh KB. 1995. Interactions between distinct types of DNA binding proteins enhance binding to *ocs* element promoter sequences. *Plant Cell* **7,** 2241–2252.

Zhu JK. 2001. Plant salt tolerance. *Trends in Plant Science* **6,** 66–71.

Zhu JK. 2003. Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology* **6,** 441–445.

Zou HF, Zhang YQ, Wei W, *et al.*. 2013. The transcription factor AtDOF4.2 regulates shoot branching and seed coat formation in Arabidopsis. *Biochemical Journal* **449,** 373–388.