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

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 5 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 exhibit distinct diurnal expression patterns and are 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 the 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 the increase of particular metabolites.

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

Introduction

DNA binding with One Finger (DOF) proteins are a group of plant-specific transcription factors (TFs) that contain a 50 amino acid 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 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 the 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 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 or plant hormone signalling (Yanagisawa, 2002, 2004a; Moreno-Risueño *et al.*, 2007a, 2007b). DOFs participate in the control of genes involved in carbon fixation and nitrogen assimilation (Yanagisawa and Sheen, 1998; Rueda-Lopez *et al.*, 2008), secondary metabolism (Skirtycz *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 (Imaizami *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 transcription factors 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-Risueño *et al.*, 2007a). *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-Risueño *et al.*, 2007a) 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 (MCOGs), A to 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, hence known as Cycling Dof Factors, CDF1-5 (Imauzumi *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). 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 that longer photoperiods cause enhanced accumulation of GI-FKF complexes and consequently decreased CDF protein levels (Imauzami *et al*., 2005; Fornara *et al*., 2009).

The *Solanaceae* family includes several horticultural crops of major economic importance, e.g. 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 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 *et al*., 2000; Apel and Hirt, 2004; Zhu 2001, 2003; 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 like 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 *Solanaceae*, like LebZIP2 (Seong *et al.*, 2008), SlAREB1 (Yañez *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 have identified 34 DOFs in tomato and performed phylogenetic analyses and comparisons with their Arabidopsis counterparts. Based on sequence similarity and domain analyses we have identified 5 genes homologous to Arabidopsis *CDFs.* 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 show improved tolerance to drought and salt when compared with the wild type. 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 the identification of DOF family members in S. 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 proteins from tomato and Arabidopsis have been further analyzed 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

ORFs of the tomato *SlCDF* genes were cloned into the pK7WGF2.0 plasmid using the Gateway recombination system (Invitrogen) to generate C-terminal GFP fusions driven by the cauliflower mosaic virus 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 ºC in the dark using a confocal microscope (LEICA-Sp2-AOBS-UV) with appropriate filters.

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

Two copies of the DOF *cis*-DNA element were produced by annealing complementary single-stranded oligonucleotides pTUYDOF-S 5´-CGTGACATGTAAAGTGAATAACGTGACATGTAAAGTGAATAA-3´ and pTUYDOF-AS 5´-CTAGTTATTCACTTTACATGTCACGTTATTCACTTTACATGTCACGAGCT-3´ that generate *XmaI* and *XbaI* cohesive ends. This fragment was cloned into the *XmaI* and *XbaI* sites of the reporter plasmid pTUY1H (Clontech) that contained 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-2xDOF were co-transfected into HF7c yeast cells. As negative control, an empty pDEST22 and pTUY1H-2xDOF vectors were used. Transformed yeast cells were plated onto SD/-Trp-Leu medium and incubated at 28 ºC. Single colonies were then streaked on SD/-Trp-Leu-His selection medium with 30 mM of 3-AT (3-Amino-1, 2, 4-triazole). The plates were subsequently incubated at 28 ºC for 2 days and yeast growth was then determined.

Protoplast transformation and GUS assays

Mesophyll protoplasts were isolated from rosette leaves of 3-week-old Arabidopsis plants (Col-0) grown in soil (21/18 ºC, 8/16 h light/dark). Protoplast isolation and transfection was performed according to the method

described by Alonso *et al,* (2009). Plasmid DNA was prepared using a Genopure Plamid Maxi Kit (Roche) and 5 µg of a pBT10-2xDOF-*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 protoplast were transferred to 2 ml microcentrifuge tubes following the procedure described in Weltmeier *et al*, (2006). 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 ºC, 16/8 h photoperiod) and irrigated regularly 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 three day-old seedlings, roots and leaves from 30 day-old plants, roots, leaves and flowers (in anthesis) from 60 day-old plants, and green (30 days after anthesis) and red (60 days 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*, three week-old uniform plantlets, bearing three leaves, were transferred to one litter plastic pots containing half strength Hoagland solution. Solutions were aerated and replaced every 4 days and plants maintained during four weeks in growth chambers (25/20 ºC; 16/8 h photoperiod). Salt stress was assayed by adding NaCl at 50 mM in the nutrient solution. PEG 8000 (Sigma) at 5% was used for water stress. Plants were transferred for 24 h to growth chambers at 35/30 ºC and 10/5 ºC, for high and low temperature stresses, respectively. Three different pools of roots and leaves were harvested (4 plants per pool) after 6, 12 and 24 h of initiating the stress. Control plants were maintained at 25/20 ºC 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 experiment (LL), plants were shifted to continuous light at dawn. After 24 h, leaves were harvested every 4 h during 24 h (0, 4, 8, 12, 16, 20 and 24 h). Three independent extracts, obtained from twelve 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 ºC until analyzed. Total RNA was extracted and purified using the 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. The ABI Prism 7000 sequence detection system (Applied Biosystems) was used for the real-time PCR with programs recommended by the manufacturer (2 min at 50 ºC, 10 min at 95 ºC, and 40 cycles of 95 ºC for 15 s and 60 ºC 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. *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 qRT-PCR analyses.

Plasmid constructs and plant transformation

The ORF of *SlCDF1* and *SlCDF3* were cloned into the Gateway binary vector pGWB2 (Nakagawa *et al*., 2007) under control of the 35S promoter. The resultant plasmid was used to transform *A. thaliana* plants, ecotype Columbia (Col-0) by the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). Transformed plants were selected on MS medium containing 50 µg/ml 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), were determined by qRT-PCR. Plants were maintained in growth chambers (21/18 ºC, 16/8 h photoperiod). Total RNA was extracted from 10 day-old seedlings to study *CO* and *FT* expression and from leaves of three-week-old plants to study *SlCDF1-3*, *COR15*, *RD29A* and *ERD10* following the protocol of Onate-Sanchez and Vicente-Carbajosa, (2008). For cDNA synthesis 2 µg of total RNA were primed with oligo dT15 primers (Promega) using the AMV Reverse Transcriptase according to the manufacturer's instructions. Arabidopsis *UBIQUITIN* mRNA level (At5g25760) was used as control. The reaction, PCR program and the analysis of the data were performed as mentioned above to analyze the expression of *CDF* genes in tomato. The primers pairs used for PCR amplification are presented in Supplementary Table S3.

Salt and drought stress tolerance tests

Salinity and drought stress assay were carried out using control plants (Col-0), *35S::SlCDF1* and *35S::SlCDF3* transgenic lines. For salinity assays, seeds were sterilized and plated onto Petri dishes containing MS medium (Murashige and Skoog, 1962). After 6 days, 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 were measured after 10 days using ImageJ software (Abramoff *et al.,* 2004). To evaluate growth differences between control and saline stress, data were represented as percentage of root growth reduction relative to standard conditions and statistical analyses were carried out by one-way ANOVA followed by Student-Newman-Keuls test ($P<0.05$). 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 days and then watering was resumed during 10 d. Plant survival rates were calculated afterwards and fresh weight was measured 10 d after re-watering period.

Metabolomic analyses

Non-targeted and targeted metabolomics analyses were performed on 12-day-old control plants (Col) and two independent *35S::SlCDF3* lines. Extraction, manipulation and mass spectrometric analysis of samples followed an adapted protocol, detailed in Supplemental file S1, 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 (Imauzumi *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, annotated and named *SlDOF1-34* (*S. lycopersicum DOFs,* Supplementary Table S1). Nucleotide sequence comparisons between genomic and cDNA clones allowed the identification of precise exon-intron structures (Supplementary Table S2). All encoded DOF proteins contain 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, B, C and 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 neighbor-joining algorithm and supported by comparisons with the Arabidopsis tree (Fig. 1A and B, Supplementary Figure S1). In both species, DOFs are clustered into four mayor groups: A, B, C and D. Three of them were further divided into subgroups based on bootstrapping values. The Arabidopsis group D1 contains the Arabidopsis *CDFs*, 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 high level of sequence similarity to the Arabidopsis CDFs. Those tomato genes have been considered as putative *CDF* orthologs from tomato and renamed as *S. lycopersicum CDF1-5,* respectively (Supplementary Table S1). This tentative asignation was further supported by the comparative analyses of the deduced amino acid sequences of the whole Arabidopsis and tomato CDFs 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 2004a; Moreno-Risueño *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 conserved in 9 of 10 sequences. These three associated motifs seem 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 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:8-12). When cells were transiently transformed with *35S::GFP*, the GFP fluorescence spread throughout the cell, indicating a cytoplasmic localization (Fig. 2A:7). Nomarski pictures (Fig. 2A:1-6) and the merged pictures of those and the fluorescence images are also shown (Fig. 2A: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 2x DOF *cis*-DNA element. Yeast growth on His-

depleted medium results from the activation of the *HIS3* gene through binding of the SlCDF proteins to the *cis*-DNA element. Addition of 3-Amino-1, 2, 4-triazole (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 later.

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-2xDOF*. The results confirmed that all of the tested CDFs can bind to the 5´-AAAG-3´ *cis*-DNA element to different extents, though, and activate the reporter gene. This shows 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*, *4* and *5*, whereas low levels were detected in those protoplasts that were transformed with *SlCDF1* and *SlCDF2*. Overall, the data obtained indicate 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.

The expression of tomato SlCDFs *follows a circadian rhythm*

To investigate whether the identified *SlCDF1-5* genes from tomato are controlled by the circadian clock like in Arabidopsis (Imauzami *et al*., 2005; Fornara *et al*., 2009), we performed quantitative qRT-PCR analyses using RNA from tomato plants grown under diurnal cycle of 16 h light/ 8 h dark (LD) 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 display quite different patterns, which could be classified in two groups (Fig. 3A and 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 its maximum level at approximately midday. Then, the expression levels 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 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 its maximum at the beginning of the light period (Fig. 3A). On the other hand, when the analyses were performed with plants grown under continuous light conditions, the expression of tomato *SlCDF1-5* genes exhibited a 24 h period oscillation pattern, which is similar to the one observed under LD (Fig. 3B). Moreover, the expression patterns of *SlCDF1-5* could still be classified into the same two groups. Taken together, these data indicate that the expression of *SlCDF1-5* is light responsive and follows a circadian pattern, which strongly supports that the identified tomato *CDF* genes are true orthologs of the Arabidopsis CDFs.

The expression of tomato SlCDF1-5 *genes is differentially regulated during development*

We analyzed the expression patterns of tomato *SlCDF1-5* genes during plant development using qRT-PCR (Fig. 3C and D) and found that *SlCDF1-5* genes have distinct patterns of expression. *SlCDF1* and *SlCDF2* show higher expression levels in vegetative compared to reproductive organs, while *SlCDF4* and *SlCDF5* are expressed at significant levels in both types. Besides, *SlCDF3* exhibits low expression in all organs analyzed. The difference in expression patterns became more evident when the expression was analyzed in closer detail during plant development (Fig. 3C). *SlCDF1, 2, 4* and *5* transcripts accumulated at high levels in cotyledons, but all of them showed minor levels of expression in mature leaves of 4-week-old plants. On the contrary, a significant increment of *SlCDF1*, *2* and *4* 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*, *4* and *5* expression was, however, reduced in roots of older plants, and no changes

were detected for *SlCDF3.* In the analyzed reproductive tissues, the expression of *SlCDF1* and *SlCDF3* was negligible when compared with the other *SlCDFs* (Fig. 3D). Higher levels of *SlCDF2*, *4* and *5* transcripts were detected in flowers, fruits and seeds. Noteworthy, during fruit ripening a considerable increment of *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 whether the expression of *SlCDFs* is also regulated by environmental cues other than light/photoperiod, *SlCDF1-5* mRNAs levels were measured in leaves and roots of three-week-old tomato plants that had been subjected to different abiotic stresses: salinity (50Mm, NaCl), osmotic (5%, PEG), heat (35/30 ºC) and cold (10/5 ºC) treatments for 6, 12 and 24 h. In leaf tissues, transcript levels of all *SlCDFs* increased under salt and osmotic stress, in particular those of *SlCDF2* and *SlCDF4* after 24 h (Fig. 4A and 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*, *3*, *4* and *5,* with decay at 24 h (Fig. 4D). Induction of *SlCDFs* 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*, *2* and *3* increased at early times (6 h) after osmotic treatment (Fig. 4A and B). Regarding to temperature treatments, maximum increase was observed for *SlCDF3* and *SlCDF5* at 24 h after heat treatment (Fig. 4C), and for *SlCDF1*, *3* and *4* at 12 h after the exposure to low temperatures (Fig. 4D).

The 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 encode proteins that

show highest sequence similarity to the functionally well-characterized Arabidopsis *CDF1* (Imazumi *et al.,* 2005; Fornara *et al*., 2009). Transgenic Arabidopsis plants overexpressing *SlCDF1* and *SlCDF3* under the control of CaMV35S promoter were generated and three homozygous lines with relatively high expression of *SlCDF*1 and *SlCDF3* were selected for further analyses (Fig. 7A). When cultured in soil under greenhouse conditions, all the overexpressing *SlCDF3* lines (L2.10, L10.4, L10.7) presented several developmental differences relative to wild-type (WT) plants (Col-0). Plants overexpressing *SlCDF3* flowered later than control plants under long day conditions but not in short day (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 both during vegetative and reproductive development. Fig. 5D, E, F, G and H exhibits representative pictures of 4-week-old WT and 35S::SlCDF3 (line 10.7 as an example) plants showing that leaves were bigger 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 significant phenotypes in the *SlCDF1* overexpressing plants (data not shown). To assess whether the late flowering phenotype observed in the *SlCDF3* overexpressing plants is 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 to 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.

The overexpression of SlCDF1 *and* SlCDF3 *has an impact in drought and salt tolerance in transgenic Arabidopsis plants*

Since our expression analyses pointed out 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 the SlCDF1 and SlCDF3. A phenotypic characterization of *35S::SlCDF1* and *35S::SlCDF3* plants was performed by analyzing their response under abiotic stresses, like 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 to wild-type plants. After 15 days of drought, plants were allowed to recover for 10 days during which they were watered. As shown in Fig. 7B, 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-days recovery period, the *35S::SlCDF1* and *35S::SlCDF*3 transgenic plants exhibited better survival and growth than the WT, as judged by their survival rates and fresh weight (Fig. 7B and 7C). To assess tolerance to salt stress, primary (PR) and lateral (LR) root elongation assays were conducted. Both *35S::SlCDF1, 35S::SlCDF3* and WT plants were grown either on control medium (w/o NaCl) or salt stress medium, containing 80 mM NaCl for 10 days (Fig. 7D and E). Under control conditions there was no difference between the transgenic and the WT plants. Only two transgenic *35S::SlCDF3* lines (10.4 and 10.7) did exhibit slightly longer roots. On salt stress media, *35S::SlCDF1-3* lines showed slight but significant reduced PR growth inhibition than the WT. Moreover the effect was more evident on LR growth, since all *35S::SlCDF1-3* transgenic plants exhibited much lower values of LR growth inhibtion than WT plants under similar stress conditions (Fig. 7D and E). Collectively, these data suggest 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 like *COR15A*, *RD29A* and *ERD10* in *35S::SlCDF1* and *35S::SlCDF3* and WT plants under control conditions. Fig. 6B shows the expression levels of the analyzed genes in transgenic lines, where they exhibited higher values (from 2 to 4

fold) than in WT plants. These data indicate 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*

Since drought and salt stress are known determinants that promote substantial physiological and metabolic rearrangements in plants (Rizhsky *et al*., 2004; Sekei *et al*., 2007), we carried out non-targeted metabolite profiling to address the question whether the ectopic expression of *SlCDF3* in Arabidopsis translates into a detectable alteration of the plants' metabolome. Principal component analysis of the retention time, intensity, and accurate mass identity matrices, carried to compare approximately 1000 molecular features per sample with each other, revealed that the overexpression of *SlCDF3* results 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 part of the differences were found among the group of small and polar compounds, containing e.g. sugars, amino acids, and small acids. As an example, the increased abundance of glutamine in the overexpressing lines compared to the wild type is shown in Fig. 8B and 8C. Hence, we focus our analyses on those polar compounds and 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 four other amino acids, eight distinct sugars plus two sugar alcohols, and eight small acids, extracted from 12-day-old WT and *35S::SlCDF3* (L2.10 and L10.7 lines) transgenic plants, grown under non-stress conditions. As shown in Fig. 8D and Supplementary Table S4, the comparison of GC profiles revealed a number of clear differences between control and overexpressing lines. Overexpression of *SlCDF3* in Arabidopsis significantly induced the accumulation of sugars like sucrose (2.5-fold), and amino acids like 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 to the wild type. Since 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 favoring 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 transcription factors that participate in different developmental and physiological processes (Lijavetsky *et al*., 2003; Moreno-Risueño *et al*., 2007a). In this work we have identified and characterized tomato *DOF* genes, homologous to Arabidopsis *CDFs* and found that the encoded proteins possess transcriptional activation ability. Furthermore, we provide 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 4 groups (A-D) on the basis of similarities in their DNA binding domains. Within group D we found 5 tomato genes with high level of sequence similarity to Arabidopsis CDFs. The encoded proteins not only show conservation in their DNA binding domain but also in their C-terminal region that contains three conserved motifs of 21, 22 and 33 amino acids, 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, 2005;

639 Sawa *et al*., 2007). In addition, these 3 motifs are also conserved in
640 homologous proteins from other species, e.g. *Jatropha curcas* (JcDOF3,
641 Yang *et al*., 2011), *Brachypodium distachyon* (BdDOF4, 11, 16, 20 and 22;
642 Hernando-Amado *et al*., 2012) and *Solanum tuberosum* (StCDF1,
643 Kloosterman *et al*., 2013). Interestingly, two allelic variants of potato *StCDF1*
644 (*StCDF1.2* and *StCDF1.3*) lacking the C-terminal end have been reported to
645 be impaired in their interaction with the FKF1-GI complex. As a
646 consequence, this results in major defects in plant maturity and tuber
647 development (Kloosterman *et al*., 2013). Consistent with these data, it may
648 be concluded that the 3 identified C-terminal motifs are common features of
649 CDF proteins, through which the regulatory mechanisms controlled by CDFs
650 are determined.

651 Subcellular localization and yeast one-hybrid assays conducted in this study
652 showed that the identified tomato SlCDFs are nuclear factors that bind to the
653 core 5'-TAAAG-3' DOF *cis*-DNA element (Yanagisawa and Schmidt, 1999)
654 with different binding affinities. Transactivation assays confirmed these
655 results and indicated that SlCDFs can act as transcriptional activators, again
656 to different extents. While SlCDF1 and SlCDF2 exhibit only little
657 transcriptional activation capabilities, SlCDF3, 4, and 5 display higher
658 transcriptional activation capacity. Consistent with these data, the
659 overexpression of *SlCDF1* and *SlCDF3* in Arabidopsis promote the
660 expression of *COR15*, *RD29A* and *ERD10*. Whether they act directly or
661 indirectly as upstream activators remain to be elucidated. In contrast, we
662 found that the overexpression of *SlCDF3* results in reduced expression of
663 both *CO* and *FT* genes, most likely acting as a target repressor, as reported
664 for the Arabidopsis CDF1 protein (Imauzumi *et al*., 2005; Fornara *et al*.,
665 2009). It should be noted that the DOF domain was at first identified as a
666 DNA-binding domain, but also reported as a bifunctional domain for DNA-
667 binding and protein-protein interactions (Mackay and Crossley, 1998).
668 Differences in the activities of DOF transcription factors have been
669 associated to the core DOF domain (Yanagisawa, 2004a) as well as their
670 protein-protein interactions with other transcription factors. In fact, the DOF
671 domain participates in the interaction with other classes of transcription

672 factors like basic domain-leucine zipper (bZIP) proteins or high-mobility
673 group (HMG) proteins, which in turn modify their transcription capabilities
674 (Vicente-Carbajosa *et al*., 1997; Yanagisawa, 1997; Zhang *et al*., 1995;
675 Krohn *et al*., 2002). For example, the Arabidopsis DOF protein OBP1 was
676 identified as a protein interacting with bZIP proteins OBF4 and OBF5
677 associated with stress responses (Zhang *et al*., 1995). Altogether, these data
678 suggest that the identified SlCDFs could display different transcription
679 activities depending on target gene promoters and the combinatorial
680 interactions with other transcription factors present in a particular tissue or
681 under different environmental conditions.

682 *The expression of* SlCDFs *follows a circadian rhythm with two different*
683 *patterns*

684 Diurnal oscillation of transcript levels of *CDFs* has been reported for
685 *Arabidopsis* and other species under day/night and constant light conditions
686 (Imaizumi *et al*., 2005; Fornara *et al*., 2009; Iwamoto *et al*., 2009; Yang *et al*.,
687 2011). *AtCDFs* exhibit different diurnal expression patterns that can be
688 classified in two different groups: *CDF1*, *2*, *3* and *5* show maximum
689 expression at the beginning of the light period, decreasing progressively
690 thereafter to a minimum between 16-20h, then rising again during dawn; and
691 the group of *CDF4*, whose transcript levels rise progressively from dawn and
692 decrease at the end of the night (Fornara *et al*., 2009). In the present study,
693 the identified tomato *SlCDFs* that exhibit similar diurnal expression patterns
694 under LD and continuous light conditions, supporting the assumption that
695 they are true homologues of the *Arabidopsis* CDFs. Interestingly, their gene
696 expression patterns could be also classified in two groups, the group of
697 *SlCDF1* and *SlCDF3* exhibit a maximum at the beginning of the day and
698 *SlCDF2, 4* and *5* that exhibit maximum levels during the night period,
699 suggesting that the family of CDFs might display different function (at least
700 two conserved functions) and regulate specific target genes at different
701 periods of the day.

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

It is well established that regulation of the temporal expression of the transcription factor CONSTANS is crucial to control the photoperiodic flowering in *Arabidopsis* and other photoperiod-sensitive species (Suarez-Lopez *et al*., 2001; Mizoguchi *et al*., 2005). The induction of *CO* mRNA by light under LDs, but not in SDs, is a key element for the triggering of flowering, since 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 (Imazumi *et al*., 2005; Fornara *et al*., 2009). Our results show that the overexpression of tomato *SlCDF3*, in analogy to *Arabidopsis CDF1*, promotes late flowering in *Arabidopsis*. Interestingly, *SlCDF3* overexpression also leads 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 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 *CDFs,* 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 as 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 *SlCDFs* respond 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 exhibit higher expression levels of abiotic stress-responsive genes, like *COR15, RD29A* and *ERD10,* under non-stress conditions, which indicate 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 (Hoekstra *et al*., 2001; Rizhsky *et al*., 2004) aiding stress tolerance through osmotic adjustment, detoxification of reactive oxygen species and intracellular pH regulation (Rajasekaran *et al*., 2000; Claussen, 2005; Munns and Tester, 2008; Bressan *et al*., 2009; Chaves *et al*., 2009). Their significant increased levels, promoted by the overexpression of *SlCDF3* in Arabidopsis, seemingly contribute to improved drought and salt tolerance since its content has been correlated with the 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.

The impact of SlCDFs *expression on C/N metabolism*

SlCDFs exhibit different expression patterns during development. However, with the exception of *SlCDF3,* all of them are 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 show that the overexpression of *SlCDF3* transcription factor in Arabidopsis results in

significant metabolic alterations. Specifically, we observed higher levels of sucrose and of certain amino acids, indicative of increased nitrogen assimilation, as previously reported for other DOF transcription factors (Yanagisawa *et al*., 2004b). In this line, our studies revealed also 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). Since carbon and nitrogen metabolites mutually influence each other in a fine balance between carbon and nitrogen metabolism (Yanagisawa *et al*., 2004b; 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 N/C balance. Hence, we hypothesize that *SlCDFs* genes could be involved in the regulation of the 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 in LDs although with a different impact in Arabidopsis (Imazumi *et al.,* 2005; Fornara *et al*., 2009). Here, we could demonstrate conservation in 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, data presented here reveal 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 show important changes in particular metabolites, like increased levels of sucrose and certain amino acids, typically associated to physiological states like the nutrient salvage and recycling under senescence programs (Jones, 2013) or the mobilization and relocation of resources from source to sink organs. This information opens 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 material

Supplementary data are available 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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Figure legends

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 the 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), respectively. The resulting groups are shown as A, B, C or D and subscript numbers indicate defined subgroups. The scale bar corresponds to 0.05 estimated amino acid substitution 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 groups D of the phylogenetic trees. Position of the identified motifs is relative to the DOF domain. Multilevel consensus sequences for the MEME defined motifs are listed.

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-SlCDFs) 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 (LEICA-Sp2-AOBS-UV) for the emission spectrum of the GFP (7-12) or by Nomarski (1-6). Merged Nomarski and fluorescence images (13-18). Arrows point to cell nuclei. **(B)** The DNA binding specificity of SlCDF1-5 proteins was assayed using the yeast one-hybrid system. Yeast HF7c cells were transfected with the genes encoding SlCDF proteins and pTUY1H driving *HIS* expression under the control of 2xDOF binding element. The transformed yeast cells were plated onto the SD/−His/−Trp/−Leu medium including the indicated amounts 3-amino-1, 2, 4, -triazole (3-AT). Empty pDEST22 plasmid was used as 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 2X DOF cis-DNA element. Empty pK7WGF2.0 plasmid was used as negative control. Data are expressed as means ± standard errors of three independent experiments.

Fig. 3. Transcription analyses of tomato *SlCDF1-5* genes during development and in response to different light conditions. **(A-B)** *SlCDF1-5* gene expression analyzed by qRT-PCR in 7-week-old tomato plants grown under 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 *SlCDFs* genes. *SlCDF1-5* gene expression was analyzed by qRT-PCR using RNA extracted from vegetative and reproductive tissues of tomato: radicles and cotyledons from three day-old seedlings (root 3d and cotyledons, respetively), root and leafs from 30- and 60-day-old plants (root 30d, 60d, leaf 30d, 60d, respectively) imbibed seeds (seed), flowers from 60-day-old plants (flower 60d), green and red fruit 30 and 60 days after anthesis, respectively (green and red fruit, respectively). Expression of tomato *UBIQUITIN3* gene (Hoffman *et al*., 1991) was used as reference gene. Data (A-D) are expressed as means ± standard errors of three independent pools of extracts. Three technical replicates were performed for each extract.

Fig. 4. Transcription analysis of tomato *SlCDF1-5* genes analyzed 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)**, 5% PEG 8000 for drought stress **(B)** exposed to 35/30 °C for high temperature stress **(C)** or exposed 10/5 °C for low temperatures stress, for the indicated times **(D)**. Expression of tomato *UBIQUITIN3* gene (Hoffman *et al*., 1991) was used as reference gene. Results are presented as relative expression of *SlCDF1-5* under stress conditions compared to the expression under control conditions. Data (A-D) are expressed as means ± standard errors of three independent pools of extracts. Three technical replicates were performed for each extract.

Fig. 5. Phenotypic differences of Col-0 and *35S::SlCDF3* plants during vegetative and reproductive development. **(A)** Representative images of four-

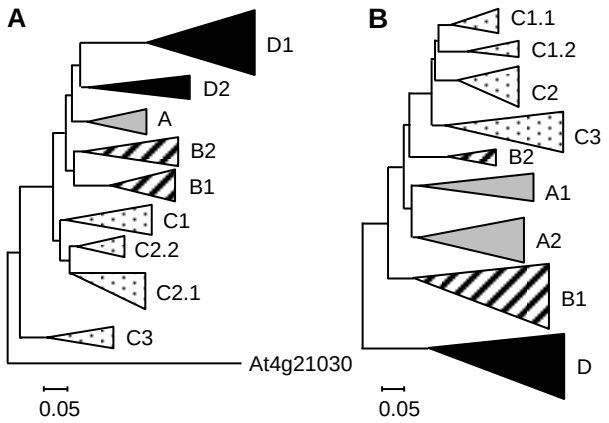
week-old plants WT and *35S::SlCDF3* (L10.7) grown under LD. **(B-C)** Flowering-time phenotype under long day (LD) and short day conditions (SD), respectively. **(D)** Rossete leaves of Col-0 and *35::SlCDF3* plants grown under LD conditions. All leaves, including cotyledons, are shown in order of production 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)** Wild type and *35S::SlCDF3* flower gynoecium. **(I)** Col-0 and *35S::SlCDF3* siliques. **(J)** Flowering time analyses of Col-0 and *35S::SlCDF3* (L2.10, L10.4, L10.7) lines estimated as rosette leaf number formed under LD conditions. Data are expressed as means ± standard errors of 20 homozygous plants. Different letters indicate significant differences ($P<0.05$; one-way ANOVA followed by Student-Newman-Keuls).

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 analyzed by qRT-PCR in *35S::SlCDF3* (L2.10, L10.7) and control plants (Col-0). Total RNA was extracted from 10-day-old seedlings and harvested, at the indicated times, throughout a long day. White and black bars along the horizontal axis represent light and dark period, respectively. **(B)** The expression of *COR15*, *RD29A* and *ERD10* genes was analyzed by qRT-PCR on three-week-old *35S*::*SlCDF1* (L1.2, L1.4, L2.6), *35S*::*SlCDF3* (L2.10, L10.4, L10.7) and control (Col-0) plants. Expression of Arabidopsis *UBIQUITIN10* gene (Czechowski *et al*., 2005) was used as reference gene. Data (A-B) are expressed as means ± standard errors of three independent pools of extracts. Three technical replicates were performed for each extract.

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* (L1.2, L1.4, L2.6) and *35S::SlCDF3* (L2.10, L10.4, L10.7) transgenic lines. *SlCDF1-3* expression was analysed by qRT-PCR in Arabidopsis plants. Expression of Arabidopsis *UBIQUITIN10* gene (Czechowski et al., 2005) was used as reference gene. Data are expressed as means ± standard errors 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 two-week-old *35S::SlCDF1* (L1.2, L1.4, L2.6), *35S::SlCDF3* (L2.10, L10.4, L10.7), and control (Col-0) plants, that were maintained 15 days without irrigation and then 10 days of re-watering. Representative images of plants before and after the treatment. Survival rates are indicated under the photographs. **(C)** Fresh weight data are expressed as means ± standard errors 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 Student-Newman-Keuls). **(D)** Salt stress tolerance estimated by determining the reduction of PR and LR growth of *35S::SlCDF1* (L1.2, L1.4, L2.6), *35S::SlCDF3* (L2.10, L10.4, L10.7) and control (Col-0) plants after 10d in MS supplemented with 80mM NaCl and represented as percentage of reduction relative to standard conditions. Data are expressed as means ± standard errors 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 ($P<0.05$; ANOVA Student-Newman-Keuls tests. **(E)** Representative images of Col-0, *35S::SlCDF1* (L2.6) and *35S::SlCDF3* (L2.10) after the treatments.

Fig. 8. Metabolic analyses of *35S::SlCDF3* and WT plants. **(A)** Principal component analysis of recorded, non-targeted metabolic profiles using Profile Analysis (Bruker Daltonics, Bremen, Germany). Projection plots obtained for principal component 1 (PC1, 19% variance explained) and PC2 (15%). Distinct grouping supports the different genotypes analyzed: wild type control samples (WT) 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 wild type) of selected metabolites analyzed by GC-SIM-MS. Given are means ± SE (n = 15). Similar results were obtained in five independent experiments. [Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$].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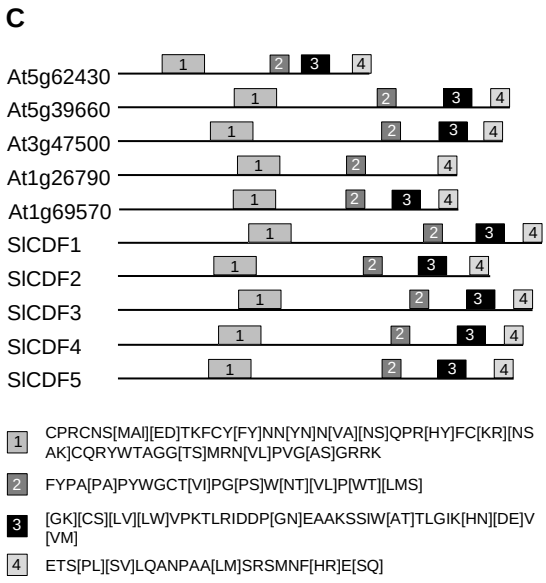
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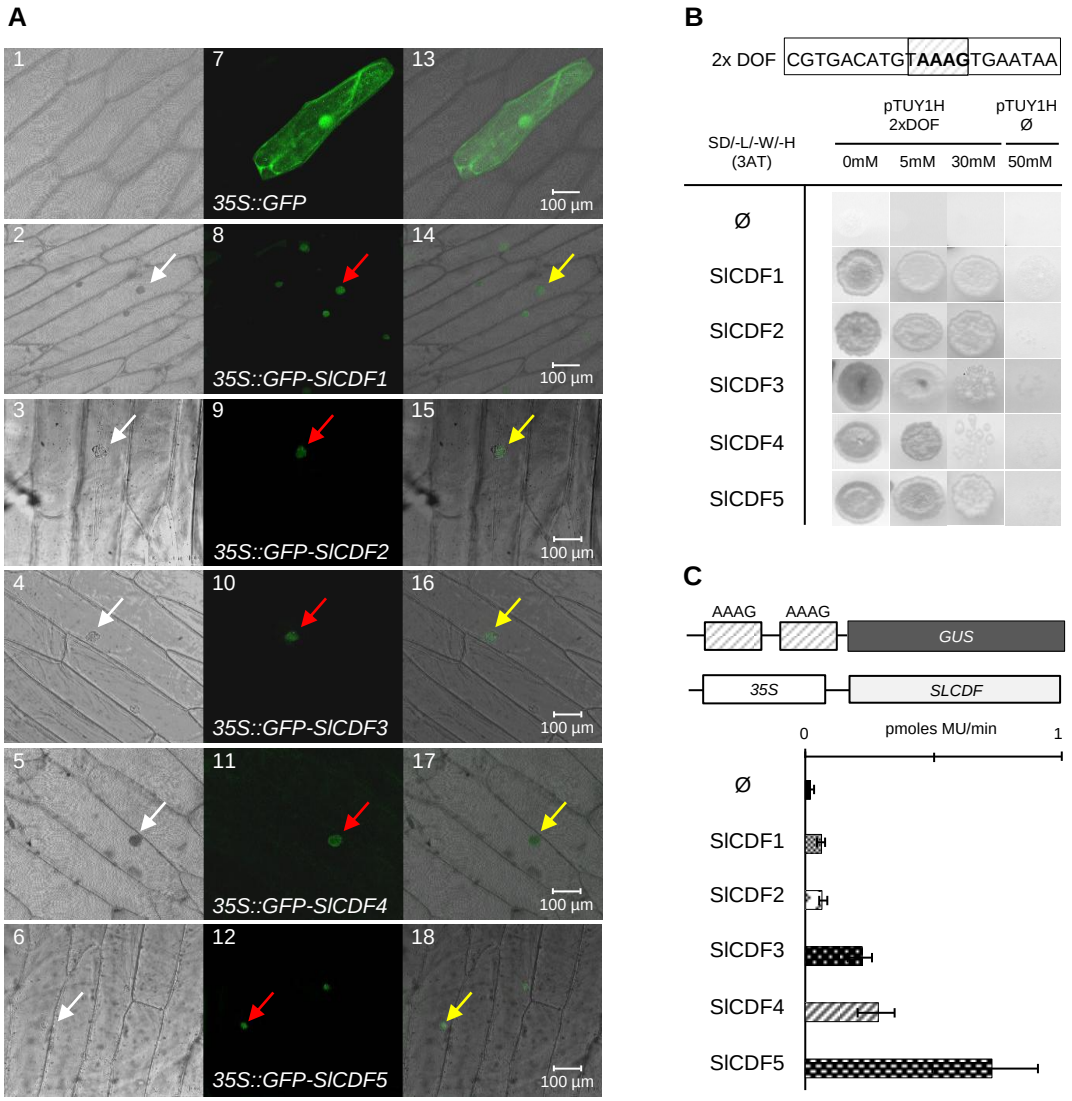
1 CPRCNS[MAI][ED]TKFCY[FY]NN[YN]N[VA][NS]QPR[HY]FC[KR][NSAK]CQRYWTAGG[TS]MRN[VL]PVG[AS]GRRK
2 FYPA[PA]PYWGCT[VI]PG[PS]W[NT][VL]P[WT][LMS]
3 [GK][CS][LV][LW]VPKTLRIDDP[GN]EAAKSSIW[AT]TLGIK[HN][DE]V[VM]
4 ETS[PL][SV]LQANPAA[LM]SRSMNF[HR]E[SQ]

Figure 1.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10
16
35S::GFP-SlCDF3
100 µm
5
11
17
35S::GFP-SlCDF4
100 µm
6
12
18
35S::GFP-SlCDF5
100 µm
B
2x DOF CGTGACATGTAAAGTGAATAA
pTUY1H 2xDOF
pTUY1H Ø
SD/-L/-W/-H (3AT)
0mM
5mM
30mM
50mM
Ø
SlCDF1
SlCDF2
SlCDF3
SlCDF4
SlCDF5
C
AAAG
AAAG
GUS
35S
SLCDF
0
pmoles MU/min
1
Ø
SlCDF1
SlCDF2
SlCDF3
SlCDF4
SlCDF5

Figure 2.

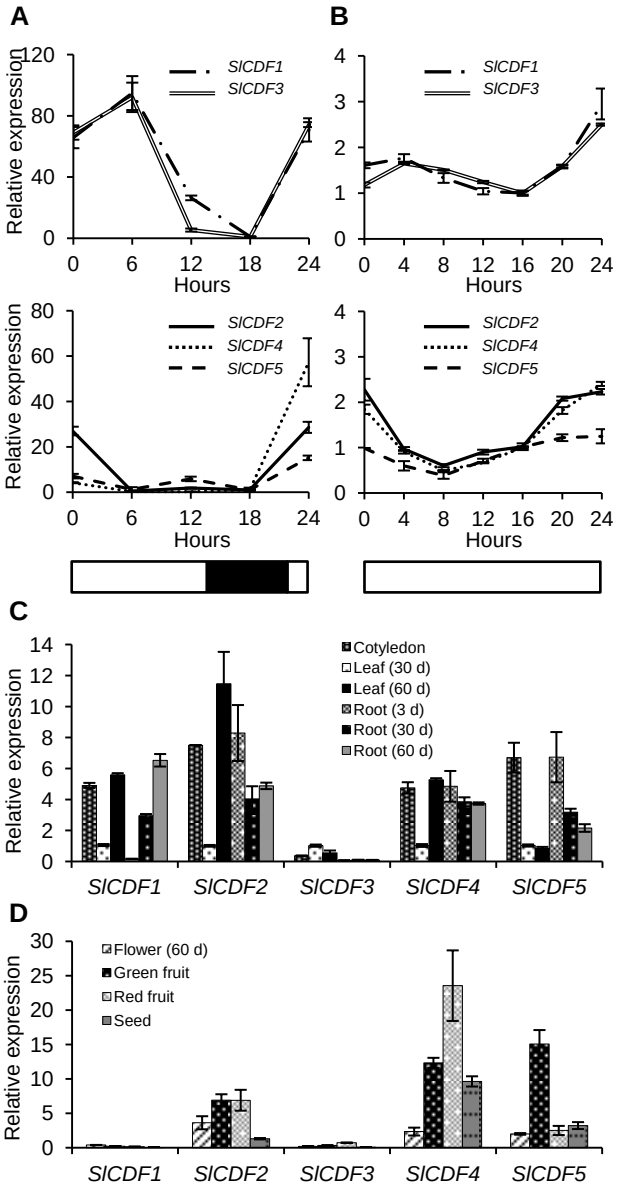
A
B
Relative expression
SlCDF1
SlCDF3
SlCDF2
SlCDF4
SlCDF5
Hours
C
Cotyledon
Leaf (30 d)
Leaf (60 d)
Root (3 d)
Root (30 d)
Root (60 d)
SlCDF1
SlCDF2
SlCDF3
SlCDF4
SlCDF5
D
Flower (60 d)
Green fruit
Red fruit
Seed

Figure 3.

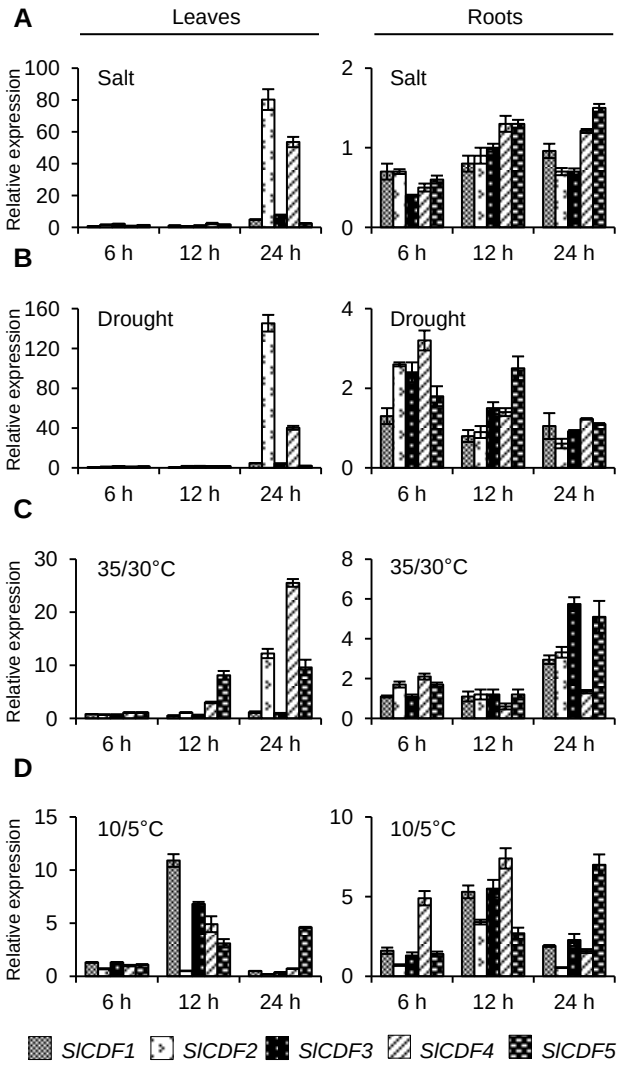
A
Leaves
Roots
Relative expression
Salt
100
80
60
40
20
0
2
1
0
6 h
12 h
24 h
B
Drought
160
120
80
40
4
2
C
35/30°C
30
20
10
8
6
D
10/5°C
15
5
SlCDF1
SlCDF2
SlCDF3
SlCDF4
SlCDF5

Figure 4.

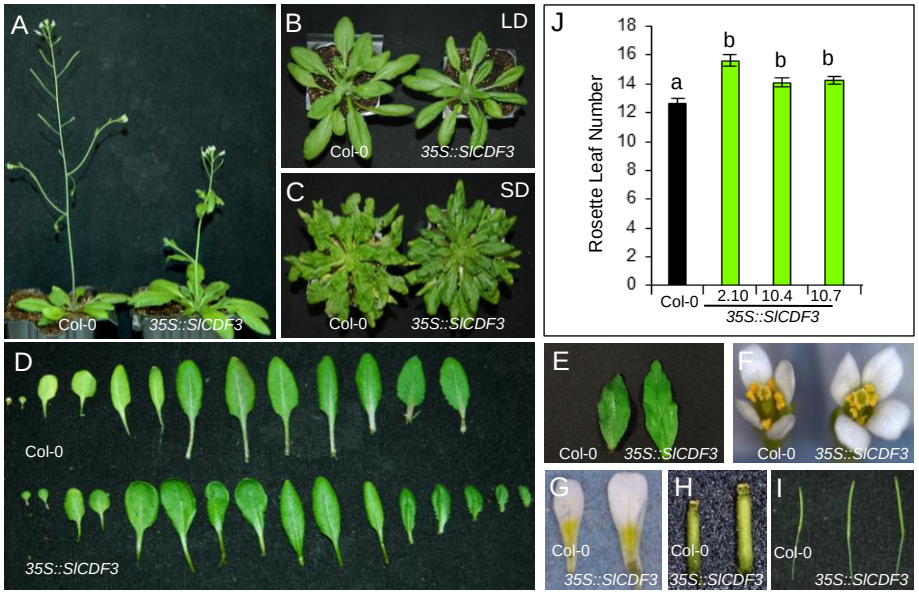
A
Col-0
35S::SlCDF3
B
LD
Col-0
35S::SlCDF3
C
SD
Col-0
35S::SlCDF3
J
Rosette Leaf Number
18
16
14
12
10
8
6
4
2
0
a
b
b
b
Col-0
2.10
10.4
10.7
35S::SlCDF3
D
Col-0
35S::SlCDF3
E
Col-0
35S::SlCDF3
F
Col-0
35S::SlCDF3
G
Col-0
35S::SlCDF3
H
Col-0
35S::SlCDF3
I
Col-0
35S::SlCDF3

Figure 5.

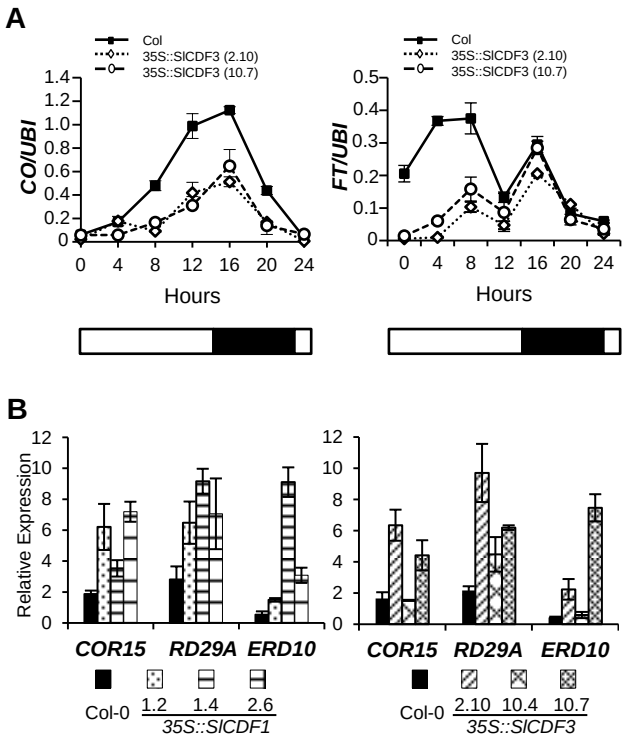
A
Col
35S::SlCDF3 (2.10)
35S::SlCDF3 (10.7)
CO/UBI
FT/UBI
Hours
B
Relative Expression
COR15
RD29A
ERD10
Col-0
1.2
1.4
2.6
35S::SlCDF1
2.10
10.4
10.7
35S::SlCDF3

Figure 6.

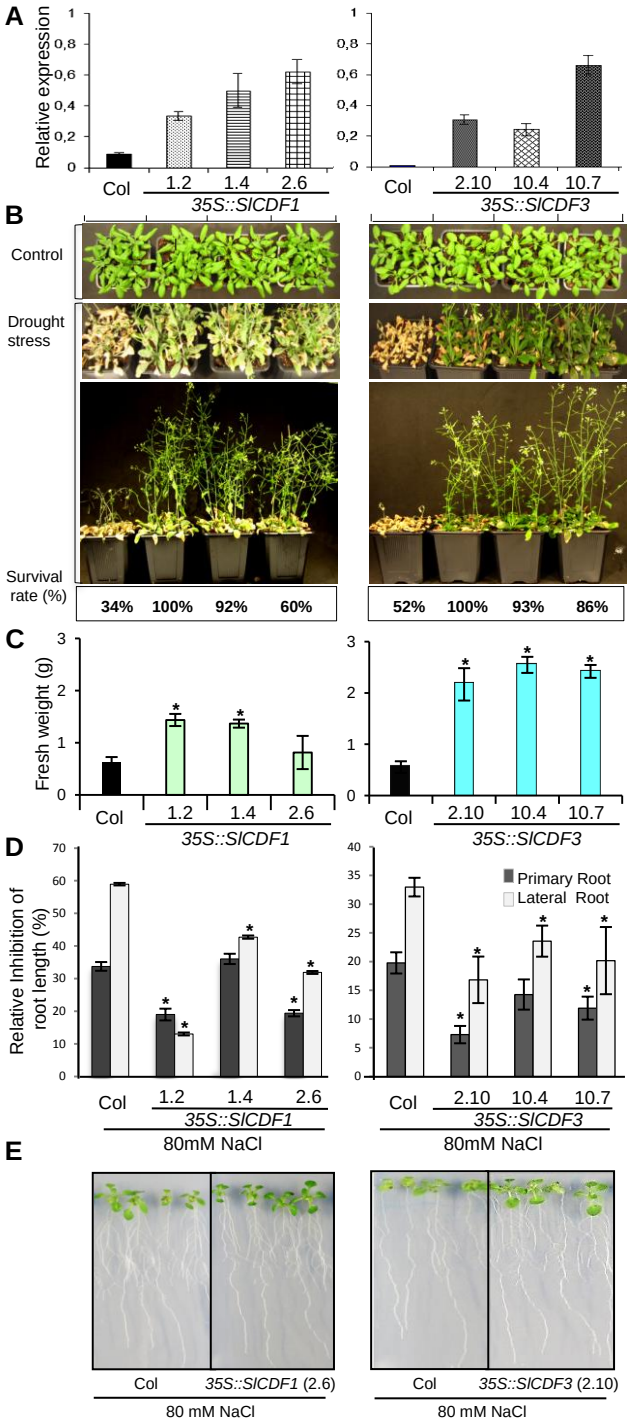
A
Relative expression
Col
1.2
1.4
2.6
35S::SlCDF1
Col
2.10
10.4
10.7
35S::SlCDF3
B
Control
Drought stress
Survival rate (%)
34%
100%
92%
60%
52%
100%
93%
86%
C
Fresh weight (g)
Col
1.2
1.4
2.6
35S::SlCDF1
Col
2.10
10.4
10.7
35S::SlCDF3
D
Relative Inhibition of root length (%)
Primary Root
Lateral Root
Col
1.2
1.4
2.6
35S::SlCDF1
80mM NaCl
Col
2.10
10.4
10.7
35S::SlCDF3
80mM NaCl
E
Col
35S::SlCDF1 (2.6)
80 mM NaCl
Col
35S::SlCDF3 (2.10)
80 mM NaCl

Figure 7.

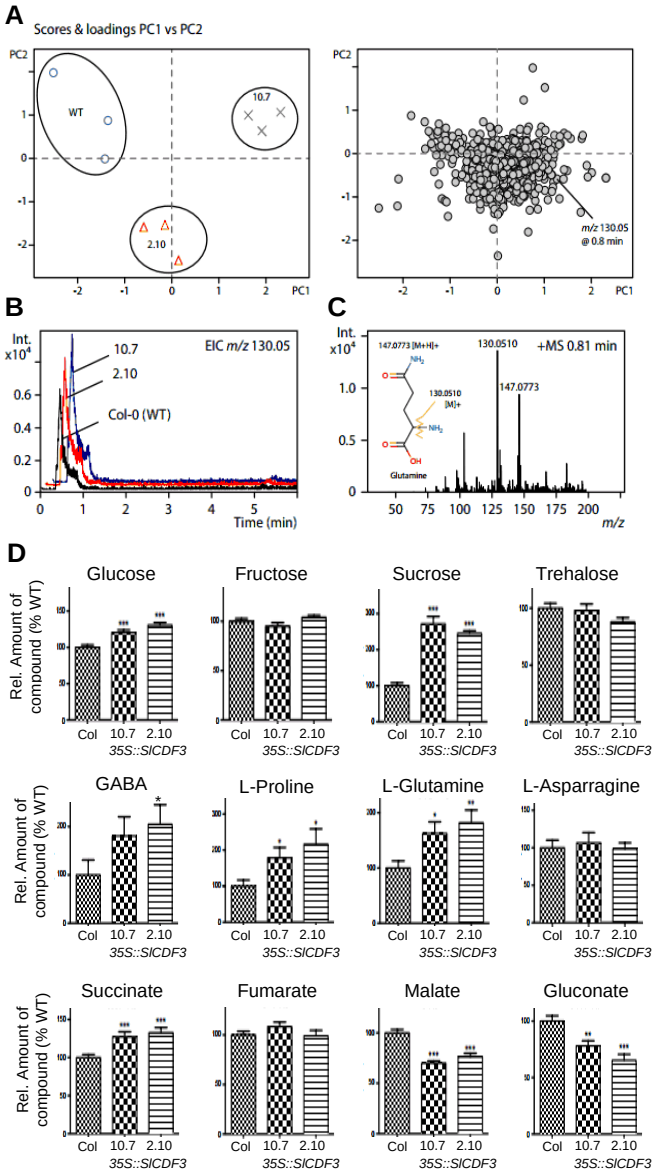
A
Scores & loadings PC1 vs PC2
PC2
PC1
WT
10.7
2.10
m/z 130.05
@ 0.8 min
B
Int.
x10^4
EIC m/z 130.05
10.7
2.10
Col-0 (WT)
Time (min)
C
Int.
x10^4
147.0773 [M+H]+
130.0510
+MS 0.81 min
130.0510
[M]+
147.0773
Glutamine
m/z
D
Glucose
Fructose
Sucrose
Trehalose
GABA
L-Proline
L-Glutamine
L-Asparragine
Succinate
Fumarate
Malate
Gluconate
Rel. Amount of compound (% WT)
Col
10.7
2.10
35S::SlCDF3

Figure 8.