Potato CYCLING DOF FACTOR 1 and its IncRNA counterpart StFLORE link tuber development and drought response

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SUMMARY

Plants regulate their reproductive cycles under the influence of environmental cues, such as day length, temperature and water availability. In Solanum tuberosum (potato), vegetative reproduction via tuberization is known to be regulated by photoperiod, in a very similar way to flowering. The central clock output transcription factor CYCLING DOF FACTOR 1 (StCDF1) was shown to regulate tuberization. We now show that StCDF1, together with a long non-coding RNA (lncRNA) counterpart, named StFLORE, also regulates water loss through affecting stomatal growth and diurnal opening. Both natural and CRISPR-Cas9 mutations in the StFLORE transcript produce plants with increased sensitivity to water-limiting conditions. Conversely, elevated expression of StFLORE, both by the overexpression of StFLORE or by the downregulation of StCDF1, results in an increased tolerance to drought through reducing water loss. Although StFLORE appears to act as a natural antisense transcript, it is in turn regulated by the StCDF1 transcription factor. We further show that StCDF1 is a non-redundant regulator of tuberization that affects the expression of two other members of the potato StCDF gene family, as well as StCO genes, through binding to a canonical sequence motif. Taken together, we demonstrate that the StCDF1–StFLORE locus is important for vegetative reproduction and water homeostasis, both of which are important traits for potato plant breeding.

Keywords: Solanum tuberosum, CYCLING DOF FACTOR, drought tolerance, IncRNA, potato tuberization, stomata, water loss rate.

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INTRODUCTION

Solanum tuberosum L. (potato) is one of the most important non-grain food crops in the world, with increasing importance for the growing economies of India and China. Potato originated in the equatorial Andean region of South America (Spooner et al., 2007), where it tuberizes in response to a short-day (SD) photoperiod. During domestication, the potato crop has also adapted to the long-day photoperiod in the northern latitudes of North America, Europe and Asia (Gutaker et al., 2019). The molecular regulation of tuberization is well understood and bears a striking similarity to the regulation of flowering in model angiosperms (Abelenda et al., 2014).

In Arabidopsis thaliana, flowering is regulated by an integrated light- and clock-dependent signalling cascade that includes proteins such as GIGANTEA (GI) and FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1), which together bind to the carboxyl terminus of CYCLING DOF FACTORS (CDFs), targeting them for degradation (Imaizumi et al., 2005; Sawa et al., 2007). Arabidopsis CDFs act redundantly on the CONSTANS (CO) promoter, which in turn is an inducer of the florigen, FLOWERING LOCUS T (FT) (Andres and Coupland, 2012). CDF proteins belong to a larger group of DNA-BINDING WITH ONE FINGER (DOF) transcription factors that bind to a consensus motif in promoters of their target genes (Yanagisawa and Schmidt, 1999; Plesch et al., 2001). Multiple tandem repeats of this motif are also present in the CO promoter, and AtCDF1 protein can repress CO by binding to these motifs (Imaizumi et al., 2005; Imaizumi and Kay, 2006).

In potato, allelic variation in the 3′ of the StCDF1 gene can lead to a truncation of the coding region, thereby
eliminating the StFKF1 binding site in the protein. The resulting lack of StFKF1-mediated ubiquitination, and subsequent degradation by the proteasome, allows the StCDF1 protein to evade normal diurnal degradation (Kloosterman et al., 2013). This transposon insertion-mediated truncation results in the indirect induction of tuberization through the constitutive repression of StCO and StSP5G genes, which together repress the transcription of the tuberigen StTP6A (Kloosterman et al., 2013; Abelenda et al., 2016). Potato plants carrying one or more of these truncated allelic variants (StCDF1.2 and StCDF1.3) become ‘early’ and long-day adapted, whereas potato genotypes with the full-length wild-type protein (StCDF1.1) are generally ‘late’, and are therefore SD dependent for tuberization (Kloosterman et al., 2013).

Beyond the regulation of flowering, CDF transcription factors have been associated with abiotic stress tolerance. Overexpression of Solanum lycopersicum (tomato) SICDF1 and SICDIF3 genes in Arabidopsis leads to higher tolerance to drought stress and salt stress (Corrales et al., 2014). Furthermore, mutations in CDF3 of Arabidopsis results in increased sensitivity to both drought and low temperatures, whereas increased expression enhances tolerance to drought, cold and osmotic stress (Corrales et al., 2017). A further level of complexity is added to this gene family by the finding that some CDF genes also encode a divergently transcribed long non-coding RNA (IncRNA; Ariel et al., 2015) that appears to act as a natural antisense transcript (NAT), regulating CDF transcription (Henriques et al., 2017).

Here, we further characterize the molecular mechanisms by which the StCDF1 locus regulates tuberization and impacts on drought tolerance in potato. We show that StCDF1 strongly binds to the DOF consensus sequence present in the promoters of StCO1, StCO2 and StCO3 genes. We also demonstrate that although CDFs in Arabidopsis show functional redundancy for the regulation of flowering, StCDF1 in potato has a non-redundant role for tuberization. In addition, we find that StCDF1 has its own natural antisense transcript (StFLORE) with antiphasic gene expression over the circadian cycle. Finally, we show that changing StFLORE expression has powerful effects on water homeostasis in plants, and that this response is due to the regulation of stomatal opening in an ABA-dependent manner.

RESULTS
StCDF1 binds to the promoter of potato CONSTANS genes
We have previously demonstrated that StCDF1 regulates the transcription of StCO genes in potato (Kloosterman et al., 2013). To gain more understanding about the sequences that the StCDF1 protein binds to, we used a protein binding microarray (PBM) to identify the consensus binding sequence for this transcription factor (Godoy et al., 2011; Franco-Zorrilla et al., 2014). For this, the StCDF1 coding region was cloned in a translational fusion to a maltose binding domain and expressed in Escherichia coli, from which protein was extracted and incubated on the PBM. Results from this experiment clearly confirmed the sequence specificity of the StCDF1 protein for the core DOF motif: AAAG (Figure 1a). We were also able to define a wider sequence consensus represented by a 9-bp sequence YWAAAGRYC motif (Figure 1b). Individual nucleotide deviations from the consensus dramatically reduce the specificity of the binding (Figure 1c). Additionally, in a genome-wide scan for the presence of the canonical StCDF1 cognate using the entire upstream region (~1.5 kb) of the annotated genes of the DM1-3 516 R44 (DM) reference genome (Potato Genome Sequencing Consortium, PGSC, 2011), the best-fit curve shows that the most abundant location of the DOF motif is relatively close to the transcriptional start site (Figure 1d) (Franco-Zorrilla et al., 2014). This finding adds biological weight to the determination of the sequence motif (Figure 1c).

We have shown previously that StCDF1 transcriptionally targets StCO1 and StCO2 (Kloosterman et al., 2013). Here, we identify an unannotated homolog StCO3 that is also regulated by circadian rhythm, with a peak during the night (Figure S1a). The three StCO genes are in a tandem repeat at chromosome 2 in potato. Using chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction (qPCR), we demonstrated the direct binding of StCDF1 to the DOF motifs present in the promoters of StCO genes in vivo (Figure 1e). These experiments clearly show a strong binding and specificity to the promoter region of the StCO1, weak binding to the StCO2 promoter and high relative levels of enrichment in the StCO3 promoter in the wild-type Solanum tuberosum group Andigenum background. Another amplified control region of the genome used as a negative control (Actin) shows no binding. The StCO3 gene is repressed in 35S:StCDF1.2 overexpression lines (Figure S1b), confirming transcriptional regulation by StCDF1. Similarly, it was previously shown that StCO1 and StCO2 were also repressed in 35S: StCDF1.2 overexpression lines (Kloosterman et al., 2013). Strikingly, we observed differences between 35S:StCDF1.2 and wild-type (WT) backgrounds in the StCO3 promoter, probably indicating some type of negative transcriptional regulation of StCDF1 by itself in the overexpression lines. Taken together, from the ChIP-qPCR and the expression studies in transgenic lines, we conclude that StCDF1 is likely to regulate all of the StCOs, with StCO1 and StCO3 promoters being the targets with the highest affinity.

StCDF1 is a non-redundant regulator of tuberization
We have previously shown that the overexpression of StCDF1.2 strongly promotes tuberization in potato and...
delays flowering in Arabidopsis (Kloosterman et al., 2013). From these experiments it may be expected that silencing StCDF1 gene expression would produce a phenotype with delayed tuberization, but only if this transcription factor acts non-redundantly on the specific downstream tuberization signal transduction pathway. To check our hypothesis,

Figure 1. StCDF1 binding specificity assessed by protein binding microarray (PBM) and chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction (qPCR).

(a) Three different secondary position weight matrices (PWM) representing obtained motifs in the experiment, with Z score indicated.

(b) Representative conserved DNA binding site motif (YWAAAGRYC).

(c) Box plot of enrichment scores (ES) of the elements indicated, determined by PBM; note the low enrichment score when the element is disrupted.

(d) Plot showing the YWAAAGRYC motif average distribution and local enrichment probability using all possible promoters of annotated potato genes as the input. The x-axis represents the distance relative to the transcription start site (TSS). The y-axis represents the enrichment score (ES).

(e) Binding interaction of StCDF1 in CO promoter through the AAAG binding motif evaluated by Chip-qPCR in WT Solanum tuberosum group Andigenum (WT) and 35S:CDF1 plants. Values are averages of three biological replicates and regions with significant enrichment above the actin negative control are indicated (*P < 0.05, **P < 0.01; Holm-Sidak multiple comparison test). The relative fold enrichment was calculated using the pre-immunized serum as a background control. A schematic description of the studied genomic region is presented below. Red dots and numbers represent the qPCR primer pairs and green vertical bars represent putative StCDF1 binding sites in the StCO1, StCO2 and StCO3 promoters. All samples were collected at ZT3 under short days.

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we made an StCDF1-specific RNAi construct and transformed it into the diploid potato clone carrying the homozygous wild-type StCDF1.1 allele (CE3027). Ten transgenic potato plants were grown in the glasshouse and periodically checked for tuberization together with untransformed controls in three replicates. The CE3027 control began to tuberize 14 weeks after planting, under long-day conditions. All plants were grown until 18 weeks after planting. Six of the 10 transgenic lines did not produce any tubers (or swelling stolons). The remaining four transgenic plants had between one and three tubers and a mean total tuber fresh weight of 9.4 g per plant, compared with an average of six tubers and a mean total tuber fresh weight of 42.9 g per plant in the control plants (Table S1). The StCDF1 RNAi plants were otherwise phenotypically normal in their growth habit and flowered at the same time as the non-transformed controls. We analysed the expression of the StCDF genes that are phylogenetically closest to StCDF1: StCDF2 and StCDF3 (Figure S2). Interestingly, although StCDF1 expression was significantly downregulated in the StCDF1 RNAi lines, the expression of both StCDF2 and StCDF3 were upregulated, compared with the controls (Figure 2a). We therefore checked the expression of these two homologues in the 35S:StCDF1.2 overexpression lines and found that StCDF2 was downregulated in a light-stable version whereas StCDF3 was markedly downregulated (Figure 2). Overall, these results showed first that StCDF1 non-redundantly promotes tuberization and second that StCDF1 appears to be a master regulator of a potato gene network also comprising other StCDF genes.

The StCDF1 locus also codes for an antisense IncRNA called StFLORE

Recently, a CDF5 IncRNA was identified in Arabidopsis, and molecular analysis showed that FLORE and AtCDF5 exhibit antiphase expression that reflects a mutual inhibitory regulation of controlling flowering time (Henriques et al., 2017). Aiming to check whether the StCDF1 locus would also encode an NAT-IncRNA, we analysed the RNA-Seq data from the DM1-3 516 R44 potato reference genome (PGSC 2011; Figure S2a). The reannotation of the strand-specific reads indicates an additional gene on its antisense strand, similar to the CDF5:FLORE NAT pair in Arabidopsis. To confirm this, we performed strand-specific cDNA synthesis using four different primers to map the 3′ end of the IncRNA transcript. PCR amplification in these four cDNA templates gave rise to only three products, allowing an approximate 3′ end mapping of the transcript into the intron of the StCDF1 gene (Figure S3b). When the transcription levels of StFLORE were tested over a 24-h time course under SDs in the diploid genotype CE3027 control, StFLORE peaked at night and showed an StCDF1-antiphase expression profile similar to FLORE expression in Arabidopsis (Figure S3c).

To visualize the spatial localization of both StCDF1 and StFLORE transcripts, we fused an AtCDF1 or StFLORE upstream promoter region (2.0 and 3.3 kb, respectively) to the β-glucuronidase (GUS) gene for histochemical localization. After incubation and staining with X-Gluc, plants carrying pStCDF1:GUS showed clear macroscopic vascular staining (Figure 3a,b). Furthermore, stomatal guard cells staining was detected also in pStCDF1:GUS (Figure 3c). No staining was detected in vascular tissue and stomata guard cells in untransformed plants CE3027 (Figure S3d). The staining of pStFLORE:GUS partially overlapped with pStCDF1:GUS, as we detected vascular staining (Figure 3d,e). In comparison with StCDF1 GUS staining, it was not possible to observe expression in stomata guard cells of pStFLORE:GUS. Thus, StCDF1 and StFLORE cellular localization overlap in vascular tissue, where the main activity of StCDF1 is controlling StCO genes and StSP6A gene expression, as expected (An et al., 2004; Sharma et al., 2016). Only StCDF1 expression was found to be located in stomatal guard cells, however, indicating a specific regulatory role in this tissue.

StCDF1 locus is involved in drought stress responses

The CDF genes have been linked to abiotic stress responses to stresses such as drought, salt and extreme temperature (Corrales et al., 2014; Fornara et al., 2015; Corrales et al., 2017). Using heterozygous potato plants carrying either StCDF1.1/StCDF1.2 or StCDF1.1/StCDF1.3, compared with the homozygous StCDF1.1/StCDF1.1 (CE3027) allelic configuration, we tested whether these potato clones show differences in tolerance to drought stress. The results show that the clones carrying a single copy of the StCDF1.3 allele were significantly less tolerant to water-limiting conditions compared with either the heterozygous StCDF1.1/1.2 or the homozygous StCDF1.1 controls (Figure 3f,g). To further understand the impact of the different StCDF1 allele combinations, we generated diploid potato genotypes that were StCDF1.2 homozygotes and StCDF1.2/StCDF1.3 heterozygotes. These plants were obtained from a cross between the diploid clones E and RH (see Experimental procedures). The presence of the homozygous StCDF1.3 allele results in very early tuberization and leads to extremely weak plants with a stunted growth habit, compared with the parental controls (Figure S3e). We also analysed plants with an StCDF1.2/1.3 allele combination and these plants had fewer branches and a smaller size than the control CE3027 (Figure S3e). StCDF1.2 carries a 7-bp insertion, whereas StCDF1.3 carries a transposon insertion of 860 bp; however, both insertions are situated in the C-terminal region of StCDF1 and both result in a very similar functional truncated protein (Kloosterman et al., 2013) (Figure 3h). We hypothesized that the
different insertions have a differential effect on StFLORE transcription, which we tested by reverse-transcriptase qPCR (qRT-PCR) using strand-specific cDNA templates of the various allelic variants from StCDF1 at zeitgeber time 9 (ZT9; peak of StFLORE expression) under SD conditions. StFLORE expression could not be detected in the StCDF1.3 homozygotes (Figure 3i), probably as a result of the 860-bp displacement of the StFLORE promoter (Figure 3g). Furthermore, heterozygotes carrying one copy of the StCDF1.3 allele showed lower StFLORE expression than the control, whereas StCDF1.1/1.2 heterozygotes showed an even higher StFLORE expression than the control (Figure 3i).
Figure 3. StCDF1 locus encodes for an antisense lncRNA called StFLORE.

(a–e) β-Glucuronidase (GUS) staining of pStCDF1:GUS (#12 and #7) and pStFLORE:GUS (#12 and #15) plants, showing StCDF1 localization in young leaf (a–b) and stomata guard cells (c), and StFLORE localization in young leaf (d–e). StCDF1 and StFLORE were both expressed in vascular tissue. Scale bars: (a) 0.5 cm; (b) 20 μm; (c) 20 μm; (d) 0.5 cm; (e) 20 μm.

(f) Plants with different allele combinations of StCDF1 under normal (left) and drought conditions (right). We show 1.1/1.2 (top) and 1.1/1.3 (below) allelic combinations for StCDF1. CE3027 was used as a control for 1.1/1.1 allele combination. n = 3 biological replicates. *P < 0.05, with respect to control.

(g) Water loss after drought-stress conditions. CE3027 was used as control for 1.1/1.1 allele combination. n = 14 biological replicates. *P < 0.05, with respect to control. For further details, see Experimental procedures.

(h) Schematic representation of the three StCDF1 alleles. The protein coding region is shown as blue boxes and the relevant domains are shaded in green (DOF domain), lilac (GI binding region) and purple (FKF binding region). Transcripts of StCDF1 and StFLORE are shown as grey arrows. The non-functional StFLORE transcript in the allelic variant StCDF1.3 is represented as a dotted line. Insertions are indicated as grey triangles (7 bp in StCDF1.2 and 865 bp in StCDF1.3). Putative promoters of StCDF1 and StFLORE are shown as grey arrowheads. StFLORE starts at 2400 bp and ends around 1320 bp from StCDF1 TSS. pStFLORE is located downstream from the stop codon of StCDF1.

(i) Real-time analysis of StFLORE expression for different StCDF1 allele compositions at ZT9 under short days; 1.3/1.3 allele composition did not show StFLORE expression. Error bars: means ± SEMs, with n = 3 biological replicates. *P < 0.05, with respect to control.

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These results indicate that the presence of the StCDF1.3 allele disrupts StFLORE expression and results in detrimental growth and reduced fitness in potato.

To ascertain the abiotic stress susceptibility of the potato StCDF1 locus, we exposed StCDF1 RNAi plants to drought stress. The StCDF1 RNAi showed a dramatic increase in drought tolerance (Figure 4a). StCDF1 RNAi had a lower water loss rate under drought stress and a lower number of tubers under both optimal water conditions and drought treatment, compared with the control (Figure 4b,c). In parallel, we grew plants overexpressing StCDF1.2, which showed a weaker phenotype even under optimal water conditions (Figure S4a). Sequence analysis of the putative StFLORE promoter reveals the presence of multiple StCDF1 binding motifs (Figure S5). We therefore also checked StFLORE expression in the StCDF1.2 overexpression plants as well as the StCDF1 RNAi plants in a 24-h time course under SD conditions. We found that the highest peak of StFLORE at ZT9 was twofold upregulated in StCDF1 RNAi plants compared with the controls (Figure 4d), consistent with the high level of drought tolerance of these plants (Figure 4a). In contrast, plants overexpressing StCDF1 showed a 30-fold decrease of StFLORE expression at both ZT6 and ZT12, compared with the control (Figure 4d).

Taken together, the expression analysis indicates a repression of StFLORE expression by StCDF1. To check whether this regulation is direct, we performed a ChIP-qPCR assay using CE3027 plants expressing a GFP-tagged StCDF1 under the phloem-specific promoter SUC2. After the ChIP assay, an enrichment of chromatin was visible in the proximal region (P1) of the transcription start site of StFLORE (Figures 4e and S5b). Enrichment values are close to those of the StCDF3 promoter, used as a positive control, and probably under similar transcriptional regulation by StCDF1. From these results, we can conclude that StCDF1 negatively regulates StFLORE by directly binding to its promoter.

Increasing StFLORE expression confers drought tolerance

To gain more insight into StFLORE function we overexpressed and knocked down StFLORE gene expression. We constructed an StFLORE transcript, driven by the cauliflower mosaic virus (CaMV) 35S promoter (35S:StFLORE) and a CRISPR-Cas9 cassette targeting 1 kb of the StFLORE promoter using four RNA guides, including putative DOF-binding motifs, proximal to the lncRNA start of transcription (Figure S5a). We found that using guides 1, 2 and 3, produced deletions of 770 and 952 bp, named ΔpStFLORE#22 and ΔpStFLORE#53 (Figure S5b). Under normal growing conditions, ΔpStFLORE plants showed a delay in development, with smaller leaves and lower heights compared with the controls, whereas overexpressing StFLORE did not show any difference compared with the controls (Figure S6a). Regarding tuberization, ΔpStFLORE plant lines showed early tuberization and a slightly higher number of tubers compared with the control (Figure S6b). 35S:StFLORE showed a decrease in tuber number and also a delay in tuberization compared with the control (Figure S6b). 35S:StFLORE and ΔpStFLORE plants were exposed to 2 weeks of moderate drought stress (see Experimental procedures). 35S:StFLORE plants were more drought tolerant and ΔpStFLORE plants were more susceptible to drought stress, compared with control CE3027 plants (Figure 5a,b). In 35S:StFLORE overexpression plants, water loss was lower compared with that of control CE3027 plants and they had the lowest number of tubers under drought stress, whereas ΔpStFLORE plants had a slightly higher water loss under drought stress compared with controls and showed no differences in tuber numbers compared with controls under drought conditions (Figures 6b and S6c).

Finally, we analysed the relative expression of StFLORE and StCDF1 transcripts in our transgenic plants during a 24-hour time course under SD conditions. We found that the 35S:StFLORE plants had a similarly high expression level of StFLORE as the StCDF1 RNAi plants, peaking at ZT9, under SDs (Figures 4d and 5c). StCDF1 expression was lower in the 35S:StFLORE plants compared with the controls (Figure 5d). In contrast, ΔpStFLORE plants showed on average a sixfold lower expression of StFLORE at ZT6 and a threefold lower expression at ZT9 and ZT12, compared with controls (Figure 5c), and a higher expression of StCDF1 at ZT0 (1.4-fold), ZT6 (13-fold), ZT9 (8-fold), and ZT12 and ZT15 (22-fold), compared with controls (Figure 5d).

In 35S:StFLORE plants, we also checked for the expression of StCDF2 and StCDF3 in a 24-h time course under SD conditions. We found that these two other StCDF family members showed slightly higher expression than the controls, which is likely linked to the lower expression of StCDF1. In ΔpStFLORE plants there was a reversed pattern, where the expression of StCDF2 and StCDF3 was slightly lower than that observed in controls (Figure S7a,b). In summary, we suggest that StCDF1 and StFLORE are subject to regulatory feedback: StCDF1 is likely to regulate StFLORE expression through the binding of DOF motifs in its promoter, whereas StFLORE acts as a NAT on the StCDF1 transcript. Furthermore, increasing StFLORE expression in 35S:StFLORE transgenics confers drought tolerance to these plants. Finally, the inverse expression pattern of StCDF2 and StCDF3, compared with StCDF1, indicates that StFLORE primarily regulates StCDF1 rather than the other two family members.

Drought response is conferred by the regulation of stomatal guard cell dynamics, impacting plant water loss

We have shown that StCDF1 RNAi and 35S:StFLORE enhanced resilience to drought. Aiming to establish the
Physiological basis of this drought tolerance, we measured stomatal behaviour. We tested the stomatal aperture under ABA treatment in our transgenic plants. Our results show that although StCDF1 RNAi and 35S:StFLORE overexpression plants respond to ABA with lower stomata aperture values, 35S:StCDF1.2 and 3pStFLORE lines were insensitive to ABA treatment (Figure 6a,b). In addition, our results revealed that StCDF1 and StFLORE also affect physiological basis of this drought tolerance, we measured stomatal behaviour. We tested the stomatal aperture under ABA treatment in our transgenic plants. Our results show that although StCDF1 RNAi and 35S:StFLORE overexpression plants respond to ABA with lower stomata aperture values, 35S:StCDF1.2 and 3pStFLORE lines were insensitive to ABA treatment (Figure 6a,b). In addition, our results revealed that StCDF1 and StFLORE also affect

Figure 4. StCDF1 knock down shows enhanced drought tolerance and increased StFLORE expression.
(a) CE3027 (1.1/1.1) untransformed control and two representative StCDF1 RNAi transgenic lines under fully watered conditions (left) and after 10 days of drought (right).
(b) Water loss percentage after drought-stress conditions in StCDF1 RNAi lines. CE3027 (1.1/1.1) untransformed was used as a control. Error bars: means ± SEMs, with n = 3 biological replicates. *P < 0.05, with respect to control. For further details, see Experimental procedures.
(c) Average number of tubers under drought and fully watered conditions in StCDF1 RNAi lines. CE3027 (1.1/1.1) untransformed was used as a control. Error bars: means ± SEMs, with n = 3 biological replicates. *P < 0.05, with respect to control.
(d) Relative gene expression of StFLORE using two representative lines of StCDF1 RNAi and 35S:StCDF1.2 during a 24-h time course under optimal water conditions in short days. Untransformed CE3027 (1.1/1.1) was used as a control. Error bars: means ± SEMs, with n = 3 biological replicates. Significant changes of gene expression compared with control CE3027 (1.1/1.1): *P ≤ 0.05.
(e) StCDF1 physically associates with StFLORE promoter detected by ChIP-qPCR in SUC2:GFP-CDF1 plants. Values are averages of three biological replicates ± SDs. Regions with significant enrichment above actin negative control are indicated (**P < 0.01; ***P < 0.001, adjusted P value, Dunnett’s multiple comparison test). The relative fold enrichment was calculated using GFP alone with transformed plants as a control. As a positive control a region with a theoretically high number of StCDF1 binding sites in the StCDF3 promoter was used. All samples were collected at ZT4 under long days. Amplicons are representative of consecutive genomic regions in the promoter, from proximal (P1) to distal (P4) StFLORE transcription start site.
Figure 5. Analysis of CRISPR/Cas9 pStFLORE plants and overexpressing StFLORE lines.

(a) CE3027 (1.1/1.1) untransformed control (left) and two representative 35S:StFLORE lines after 10 days of drought (right). Error bars: means ± SEMs, with n = 3 biological replicates.

(b) CE3027 (1.1/1.1) untransformed control (left) and two representative ΔpStFLORE transgenic lines after 10 days of drought (right). Error bars: means ± SEMs, with n = 3 biological replicates.

(c) Relative gene expression of ΔpStFLORE and 35S:StFLORE during a 24-h time course under optimal water conditions in short days. The untransformed CE3027 (1.1/1.1) from Figure 4(b) was used as a control. Error bars: means ± SEMs, with n = 3 biological replicates. Significant changes of gene expression compared with control CE3027 (1.1/1.1): *P ≤ 0.05.

(d) Expression of StCDF1 in ΔpStFLORE and 35S:StFLORE during a 24-h time course under optimal water conditions in short days. Error bars: means ± SEMs, with n = 3 biological replicates. CE3027 (1.1/1.1) untransformed from Figure 2(a) was used as a control. Significant changes of gene expression compared with control CE3027 (1.1/1.1): *P ≤ 0.05.
stomata density and size (Figures S8 and S9; Table S2). For instance, StCDF1 RNAi and 35S:StFLORE overexpression plants have higher stomatal density but smaller guard cell size, and 35S:StCDF1 overexpression plants have lower stomatal density but larger stomatal guard cells (Table S2). Moreover, previously we found that StCDF1 RNAi and 35S:StFLORE overexpression plants also have the lowest values of water loss under drought stress (Figures 4b and S6c). Taken together, these results show that our tolerant transgenic StCDF1 RNAi and 35S:StFLORE overexpression plants are able to decrease their water loss not only by responding to ABA but also through the regulation of stomata size and number.

**DISCUSSION**

Cycling DOF transcription factors are transcriptional repressors that, together with TOPELESS, regulate downstream initiators of reproductive development (Imaizumi et al., 2005; Abelenda et al., 2014; Wu et al., 2017). In potato, this includes the indirect activation of tuber development (Kloosterman et al., 2013). We show here that StCDF1 protein binds to canonical DOF motifs in the promoter regions of a tandem array of three potato CONSTITANS genes located on chromosome 2. The repression of these StCO genes, and especially of StCO1, by StCDF1 is likely to prevent the induction of an FT-like repressor (StSP5G) of the tuberigen StSP6A, making StCDF1 an indirect positive regulator of tuberization. This is similar to the situation in Arabidopsis where CDF1 also represses CO (Song et al., 2012). Unlike Arabidopsis, however, where a quadruple CDF mutant is required to exhibit early flowering and be photoperiod insensitive (Fornara et al., 2009), we show here that StCDF1 is able to affect tuberization by itself. This confirms that a single potato CDF homologue has evolved specifically to regulate tuberization.

In addition to its regulation of potato StCO and StSP5G gene expression, StCDF1 represses the expression of StCDF2 and StCDF3. We noted from an analysis of PGSC RNA-Seq data that the StCDF4 locus also has a secondary gene model that is very similar to that of StCDF1 (PGSC, 2011). Recently, Kondhare et al. (2019) showed that StCDF1 gene expression is regulated by a BEL1-like protein (StBEL5). Interestingly, we found that the KNOX-partner of StBEL5 (StPOTH1) is downregulated in 35S:StCDF1 plants, indicating complex upstream regulatory circuitry that will require further analysis.

As shown for Arabidopsis (Henriques et al., 2017), potato StCDF1 also has an IncRNA natural antisense transcript, StFLORE. In potato, the antiphasic expression pattern of
these transcripts and the binding interaction of StCDF1 in the StFLORE promoter indicates that StCDF1 modulates the expression of the StFLORE transcript. In homozygous StCDF1.3 plants, we do not detect any StFLORE transcript. The transposon insertion in this natural allele, displaces the promoter thereby disrupting the functional StFLORE lncRNA transcript, in addition to truncating the StCDF1 protein. Both natural allelic variation and engineered StFLORE promoter knockdowns (StCDF1.3 homozygotes and CRISPR-cas9 deletions of the StFLORE promoter, respectively) have a profound effect on plant fitness. Conversely, the elevation of StFLORE expression, either by a reduction of StCDF1 expression or by the overexpression of the StFLORE transcript itself, leads to an enhanced tolerance to abiotic stress. Multiple links have been found between abiotic stress tolerance and CDF gene expression, including drought, salt and temperature stresses (Corrales et al., 2014; Fornara et al., 2015; Corrales et al., 2017; Renau-Morata et al., 2017). Our data from both genetic studies of allelic variants of StCDF1 and the transgenic plants with differences in gene expression of StCDF1 or StFLORE indicate that both components are likely to be responsible for the drought-tolerance phenotype in potato. The drought-tolerance phenotype of StCDF1 RNAi and 35S:StFLORE could be a consequence of the late maturity of the plants (Spitters and Schapendonk, 1990; Aliche et al., 2019). Interestingly, a preponderance of DOF binding motifs has been detected in promoters of stomatal guard cell-expressed genes (Plesch et al., 2001); however, a direct mechanistic link of StCDF1 and StFLORE with the regulation of stomatal guard cell opening and closing remains unclear on a molecular level. Our results from stomatal density and stomatal guard cell sizes in our tolerant transgenics, 35S:StFLORE and StCDF1 RNAi plants, concur with previous studies where water deficit leads to a decrease in stomatal size (Cutler et al., 1977; Quarrie and Jones, 1977; Spence, 1987) and an increase in stomatal density (McCree and Davis, 1974; Cutler et al., 1977; Yang and Wang, 2001; Zhang et al., 2006), with stomatal density linked to water-use efficiency (Yang et al., 2007). The co-expression of StCDF1 and StFLORE in the vasculature indicates a primary role for StFLORE regulation of StCDF1 in this tissue. If not a result of technical limitations in the promoter:GUS approach, the lack of StFLORE expression in stomatal guard cells indicates that there may be further intermediates affecting the influence of this transcript, however. Significant differences in the expression of ABA biosynthesis genes in StFLORE overexpression and knockout plants, as well as the transcriptional responsiveness of StFLORE gene expression to ABA, may provide an interesting lead for this signalling pathway. Nevertheless, we present data from natural StFLORE promoter knockdowns, CRISPR-Cas-9 mutants and overexpression transgenics indicating that StFLORE regulates stomatal guard cell dynamics. There is a vast volume of literature that links lncRNAs to abiotic stress, however, only a few of them have been fully characterized (Matsui and Seki, 2019). Our results show that the expression of the StFLORE IncRNA is regulated by promoter binding by StCDF1 protein, and in turn this influences stomatal aperture and guard cell size. These effects are not linked to the earliness effect of StCDF1 truncation alone, as the StCDF1.2 variant appears not to have adverse effects on StFLORE expression or plant fitness under normal or drought-stress conditions.

Earliness of tuberization and life-cycle length are critical traits for plant breeding and agriculture, as they have profound effects on yield and production in various geographic locations. This importance is accentuated in the new true hybrid breeding programmes where life-cycle length is essential when planning to start with true-seed potato material. Potato crosses based solely on the phenotype do not guarantee avoiding early allelic variants of StCDF1 in the progeny. Moreover, knowing that a single StCDF1.3 allele produces a negative effect on fitness under abiotic stress situations, it is indispensable to develop specific molecular markers to distinguish the early alleles in breeding programmes so as to not introduce adverse effects of knocking down the StFLORE transcript.

**EXPERIMENTAL PROCEDURES**

**Potato material and growth conditions**

Potato plants with different allelic combinations of StCDF1 were obtained from C × E diploid clones. Clone C (USW5337.3) is a hybrid between Solanum phureja Pi25988.1 and S. tuberosum dihaploid USW42. Clone E (VPH4 772102.37) is the result of a backcross between clone C and Solanum vernei–S. tuberosum clone VH3 4211. RH (RH89-039-16), a diploid heterozygous potato clone, and E were crossed to obtain the 1.3/1.3 allelic combination of StCDF1. The C × E (CE3027, CE605 and CE630) and RH × E (RHE25) progeny, together with transgenic plants generated in this study, were vegetatively propagated and grown in vitro on MS medium supplemented with 2% w/v sucrose (Murashige and Skoog, 1962). Two-week-old plants were planted in soil and grown either in the glasshouse at 23°C under long days (LDs) or in controlled-environment chambers at 2°C under SDs (with 8 h of light and 16 h of dark). The plants used for this study are listed in Table S3, including CE3027, CE605, CE630 and RHE25, which possess 1.1/1.1, 1.1/1.2, 1.1/1.3 and 1.3/1.3 allelic compositions, respectively, which were used for molecular analysis.

**Drought exposure**

After 2 months of growing in the glasshouse, the C × E population and transgenic plants were divided into two treatments: optimal irrigation and drought stress. Optimal irrigation was considered to be, manual watering every 2 days, corresponding to 100% field capacity. Plants grown under drought conditions were also irrigated every 2 days, but with decreasing field capacities of 60% for 5 days and 40% for the following 5 days. In total, these plants were exposed to 10 days of drought stress. After approximately 120 days after sowing, tuber number and tuber fresh weight were measured for all
individuals grown under optimal irrigation and drought treatments. To obtain tuberization timing we check the tubers from week 10, when they begin to tuberize, onwards. To measure water loss under drought conditions, fully developed leaves of control CE3027 and transgenic plants were cut after 10 days of drought treatment and exposed at room temperature. The leaves were weighed 4 h after being cut. Water loss ($) was calculated as \( \frac{[\text{fresh weight} - \text{dry weight after 4 h}] \times 100}{\text{eight leaves under drought stress}} \) (Campo et al., 2012). To measure water loss under drought conditions, fully developed leaves of control CE3027 and transgenic plants were cut after 10 days of drought treatment and exposed at room temperature. The leaves were weighed 4 h after being cut. Water loss ($) was calculated as \( \frac{[\text{fresh weight} - \text{dry weight after 4 h}] \times 100}{\text{eight leaves under drought stress}} \).

**Physiological evaluation**

Stomatal aperture was measured as reported previously (Roelfsema and Prins, 1995; Desikan et al., 2005). Briefly, the leaves of 4-week-old plants growing in a glasshouse were cut and first submerged into MOCK solution (a buffer that favours stomata opening). After 3 h, half of the leaves were submerged in ABA (10 \( \mu M \)) and the other half was kept in the MOCK solution as a control (Eisele et al., 2016). Stomatal aperture in leaves treated with ABA and Mock solution was calculated by measuring width over length from the stomata aperture. The reading was performed for a total of 30 stomata in four leaves per genotype under the microscope, with a 40 \( \times \) magnification. Furthermore, we calculated stomata density as stomata number per mm\(^2\) area and stomata size by using the oval area formula (\( \mu m^2 \)).

**Histological analysis**

For the pStFLORE::GUS construct, a 3.3-kb fragment upstream of the 5\(^\prime\) transcription start site of SiFLORE (for further details of the exact position, see Table S4) was amplified by using 5\(^\prime\)-CACCTCT-CATAAGTGGAAGTAACTTCAAGA-3\(^\prime\) and 5\(^\prime\)-TCACTAAT-TATGTTGCTCATCCT-3\(^\prime\) guides. StFLORE::GUS constructs were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into diploid potato CE3027 and Solanum tuberosum, respectively. The subcellular localization of transgenic lines were used for GUS staining, as described previously (Jefferson et al., 1987). As a negative control, untransformed CE3027 was stained with GUS. Photos were taken of 3-week-old transgenic individuals grown under optimal irrigation and drought treatments. To obtain tuberization timing we check the tubers from week 10, when they begin to tuberize, onwards. To measure water loss under drought conditions, fully developed leaves of control CE3027 and transgenic plants were cut after 10 days of drought treatment and exposed at room temperature. The leaves were weighed 4 h after being cut. Water loss ($) was calculated as \( \frac{[\text{fresh weight} - \text{dry weight after 4 h}] \times 100}{\text{eight leaves under drought stress}} \) (Campo et al., 2012). To measure water loss under drought conditions, fully developed leaves of control CE3027 and transgenic plants were cut after 10 days of drought treatment and exposed at room temperature. The leaves were weighed 4 h after being cut. Water loss ($) was calculated as \( \frac{[\text{fresh weight} - \text{dry weight after 4 h}] \times 100}{\text{eight leaves under drought stress}} \).

**Generation of constructs and transformation**

RNAi lines (StCDF1#7 RNAi; and StCDF1#13 RNAi) were constructed by cloning the StCDF1 cDNA fragment using 5\(^\prime\)-CACC ATGTGCTCGAGATGATCCCCTGCT-3\(^\prime\) and 5\(^\prime\)-GACACGACAGACC GCTATG-3\(^\prime\) that contained the full coding sequence (Table S4). The inverted repeat is assembled in the binary vector by a two-step cloning process with specific restriction enzyme sites (Karimi et al., 2002). The fragment was subsequently recombined into the binary vector pK7WGIIWG2 using LR clonase (Invitrogen, now ThermoFisher Scientific, https://www.thermofisher.com) and transformed into TOP10 E. coli cells. The presence of the insert was confirmed by PCR amplification and the direction was verified by digestion and sequencing. Successful constructs were then transformed by electroporation into Agrobacterium tumefaciens GV3101 and confirmed by PCR before genetic transformation.

A plasmid with the specific SUC2 promoter was generated to obtain plants expressing GFP-CDF1.1 in the phloem. pALLIGATOR2pSUC2UTR_GW, kindly provided by G. Coupland, was used as a template to amplify the SUC2 promoter with primers including HindIII and XbaI restriction sequences. The PCR product was cloned in pGEMTeasy, sequenced, digested with HindIII and XbaI, and ligated again in pGBW406 after digestion and excision of its S5S promoter with the same enzymes, obtaining the pGBW06SUC2 plasmid. Finally, LR recombination between StCDF1.1 ORF in TOP10 pENTRD and pGBW06SUC2 was performed, and the final SUC2::GFP-SCDF1.1 plasmid transferred to CE3027 plants. In parallel, an empty SUC2::GFP-Stop construct was created to transform plants and use them as negative controls.

The CRISPR/Cas9 knockout of the StFLORE promoter was performed by using Golden Gate cloning. We selected four sgRNAs along 1.5 kb of the StFLORE promoter including StCDF1 binding sites (see Figure S5). The four sgRNA scaffold clones with 20-bp target sequences were previously obtained by PCR using a pair of synthetic specific primers using gRNA_GFP_T1 as a template. They were recombined with pCH47732:Nosp:NTII-OCST, pCH47742:35Sp:Cas9-NOST, the linker pCH47180, and cloned in pAGM4723 in a single-cut ligation reaction with Bsal and T4-ligase. (ThermoFisher Scientific). The final binary plasmid contained an hCas9 gene under the CaMV 35S promoter and four single-guide RNAs (sgRNAs) under the control of the AtU6 promoter.

To detect mutagenesis, we amplified by PCR using DreamTaq DNA polymerase (ThermoFisher Scientific), with fragments containing guides sg1, sg2, sg3 and sg4 with the forward primer 5\(^\prime\)-TCCCTTCTACTCTGACTACCTC-3\(^\prime\) and reverse primer 5\(^\prime\)-CTA- CACCAGCTTGTTGATACTGATAAATAAAAAT-3\(^\prime\) and 5\(^\prime\)-GATTGGAAGAAAGAAGGAGTTT-3\(^\prime\) (Table S4). From 100 regenerated plants we obtained two transgenic plants (\( \Delta pSIFLORE\#22 \) and \( \Delta pSIFLORE\#53 \)) from which we amplified the 1.5-kb region upstream of the transcription site of StFLORE to clone into pGEMT Easy vector (Promega, https://www.promega.com), and 10 colonies from each transgenic plant were sent for sequencing with M13 primers. In all the plants tested, at least one colony carries the intact StFLORE promoter. From these transgenics, we checked that the StCDF1 gene was not affected by our guides.

Overexpression plants of StFLORE (35S:StFLORE#207 and 35S:StFLORE#249) were constructed by amplifying the reverse complement from the StCDF1 gene with the forward primer 5\(^\prime\)-CACCGGAGAGTGTAGAGAGATCTGATAAGAGTAGATT-3\(^\prime\) and reverse primer 5\(^\prime\)-CTA CACTCTCTCAGATCCCATTTG-3\(^\prime\) (Table S4). The PCR-generated full-length sequence was cloned into pENTR TOPO vector and TOP10E cells were transformed according to the manufacturer’s instructions (Invitrogen, now ThermoFisher Scientific). An LR recombination reaction was performed between the entry clone (pENTR TOPO) and the destination vector (pK7WG2), according to the manufacturer’s protocols (Invitrogen, now ThermoFisher Scientific). Transgenic plants overexpressing StCDF1.2 (35S: StCDF1#3 and 35S:StCDF1#10) were described previously (Kloosterman et al., 2013).
RNA extraction and qRT-qPCR analysis

The samples were harvested and stored in liquid nitrogen until RNA extraction. The isolation of total RNA was performed with the RNeasy mini kit (Invitrogen, now ThermoFisher Scientific) following the corresponding protocol. DNA digestion was accomplished by DNase I (TaKaRa, https://www.takarabio.com), and the first-strand cDNA was obtained according to the manufacturer’s instructions, by either superscript VI reverse transcriptase (Invitrogen, now ThermoFisher Scientific) to quantify StFLORE expression or by iScript cDNA synthesis kit to quantify StCDF1, StCDF2 and StCDF3. The NAC gene was chosen as the housekeeping gene, and qRT-PCR was performed using SYBR green MasterMix (Bio-Rad, https://www.bio-rad.com) on a real-time PCR System (CFX96; Bio-Rad) with the specific primers listed in Table S4. The qRT-PCR programme consists of 95°C for 3 min and 42 cycles of 95°C for 5 sec and 60°C for 10 sec. The relative expression level of each examined gene was quantified by a relative quantification method.

ChIP-qPCR analysis

Chromatin immunoprecipitation (ChIP) on the StCOs cluster was performed as described elsewhere, with minor modifications (Abelenda et al., 2016). We used a specific StCDF1 antibody in a ChIP experiment on nuclear extracts from WT S. andigena plants and transgenic S. andigena overexpressing StCDF1,2 from CaMV 35S promoter (p35SStCDF1.2). A preliminary step of incubation and chromatin clean-up with the pre-immunized serum was included. After incubation with the antibody, chromatin isolation using G protein coupled to paramagnetic beads (Dynabeads Protein G; Novex Life Technologies, now ThermoFisher Scientific) was performed. After reverse crosslinking, chromatin was recovered by column purification using the QiAquick PCR clean up Kit (Qiagen, https://www.qiagen.com). ChIP-qPCR was assayed with specific primers to quantify StCDF1 affinity for different StCO1–StCO2 DNA binding sites, and the enrichment of eight separate amplified regions of the StCO gene cluster was quantified. Regarding StCDF1 binding detection in the StFLORE promoter by ChIP, SUC2:GFP-CDF1.1 transgenic plants in the CE3027 background were used. Plants were grown under LDs for 3 weeks, and material was collected at ZT4. Rabbit polyclonal anti-GFP antibody (Ab290; Abcam, https://www.abcam.com) was used in combination with Dynabeads Protein G and protein A (50/50 V/V). Control plants expressing GFP alone were used as a control. Four different regions were assayed by qPCR. Primer sequences are listed in Table S4. Regarding StCDF1 binding detection in the StFLORE promoter by ChIP, SUC2:GFP-CDF1.1 transgenic plants in the CE3027 background were used. Plants were grown under LDs for 3 weeks, and material was collected at ZT4. Rabbit polyclonal anti-GFP antibody (Ab290; Abcam) was used in combination with Dynabeads Protein G and protein A (50/50 V/V). Control plants expressing GFP alone were used as a control. Four different regions were assayed by qPCR. Primer sequences are listed in Table S4.

All ChIP enrichment calculations were performed using the SuperArray ChIP-qPCR Data Analysis Template (Qiagen/SABioscience), following the manufacturer’s instructions.

Protein binding microarray assay and analysis

Recombinant MBP-StCDF1 protein was obtained in E. coli Rosetta™ strain (Novogen, https://novogen-layers.com) after cloning in pMAL-c2 vector (New England Biolabs, https://www.
**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** StCDF1 acts a repressor of StCO3.

**Figure S2.** StCDF1 knockdown non-redundantly delays tuberization.

**Figure S3.** The StCDF1 locus encodes for an antisense IncRNA called StFLORE.

**Figure S4.** StCDF1 knockdown shows enhanced drought tolerance.

**Figure S5.** Analysis of CRISPR/Cas9 pStFLORE plants and overexpressing StFLORE lines.

**Figure S6.** Analysis of CRISPR/Cas9 pStFLORE plants and overexpressing StFLORE lines.

**Figure S7.** Analysis of CRISPR/Cas9 pStFLORE plants and 35S:StFLORE lines.

**Figure S8.** Effect of StCDF1 expression on stomatal response to ABA treatment.

**Figure S9.** Effect of StFLORE expression on stomatal response to ABA treatment.

**Table S1.** Summary of the tuberization data of control (CE3027), StCDF1 RNAi and overexpressing StCDF1:2 independent lines.

**Table S2.** Summary of average stomatal features from StCDF1 RNAi, 35S:StCDF1, 35S:StFLORE, 35S:StFLORE and CE3027, as a control.

**Table S3.** List of plants from the C x E population used in this study.

**Table S4.** Oligonucleotides used in this study.

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