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Circadian-regulated *lncCDF1* and its natural antisense transcript *CDF1* constitute a novel regulatory module to control drought stress responses in *Arabidopsis thaliana*

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for the degree of

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ABSTRACT

Drought is a critical abiotic stress that has significant impacts on plant growth and productivity. In recent years long non-coding RNAs (lncRNAs) have emerged as key regulators of plant processes, with direct involvement in developmental transitions (e.g., germination, flowering time, senescence), or in the responses to abiotic stress conditions (e.g., cold, drought). Here I investigated a novel circadian lncRNA, *lncCDF1*, which is a natural antisense transcript (NAT) to *CYCLING DOF FACTOR 1* (*CDF1*). Previous studies showed that *lnccdf1* mutants performed better under drought stress conditions, suggesting a role for this lncRNA in mediating these responses. To further characterise this phenotype, we examined flowering time of wildtype (Col-0), *lncCDF1* overexpressor lines, the *lnccdf1* knockdown mutant, the *cdf-quadruple mutant* and the *HA-CDF1* overexpressor line grown under control and drought conditions. Our findings showed that *lncdf1* mutant had a late-flowering phenotype, whereas *lncCDF1* overexpressors displayed early flowering. In parallel, we performed genome-wide transcriptional profiling of wildtype (Col-0), *lncCDF1* overexpressors and mutant, and *HA-CDF1* overexpressing line to determine the expression of *CDF1/lncCDF1* and five drought-responsive genes. Three of these genes; *SUC7*, *OSM34* and *MFT* displayed upregulated transcription under drought conditions in the *lnccdf1* mutant. The other two genes *QQS* and *EXT1* exhibited altered responses to drought stress in *lncCDF1* transgenic lines. Finally, we performed starch and soluble sugar analysis to assess diurnal sugar metabolism in *lncCDF1* transgenic lines under drought conditions. Our results indicate that *lncCDF1* alters starch and soluble sugar metabolism especially at ZT6 and ZT16. Notably, we observed an increase of soluble sugars in the *lncCDF1* overexpressors under drought conditions at ZT6. Moreover, under control conditions the *lnccdf1* mutant accumulated less soluble sugars compared with wildtype, but drought treated plants displayed a consistent increase across all timepoints. Considering that *lncCDF1* modulates *CDF1* transcription and waveform we propose that this novel NAT pair could potentially coordinate developmental timing and metabolic stress responses, highlighting new insights into lncRNA-mediated drought tolerance.

Keywords: drought, long non-coding RNA, transcriptomic profiling

1.0 Introduction

1.1 Circadian Regulation and Plant Stress Responses

As plants are sessile organisms they have evolved mechanisms to cope with unfavourable conditions and the daily changes in light-dark cycles influenced by the rotation of the earth (Sanchez and Kay, 2016). The circadian clock provides plants with the ability to synchronize their metabolism, physiology and development, to daily and seasonal environmental changes. Plants can then anticipate changes in their surroundings by generating endogenous 24-h rhythms of physiological responses in tune with external and internal cues (Xu *et al.,* 2022). The clock is the result of transcriptional and translational feedback loops dominated by repressive interactions (Linde *et al.,* 2017).This photomorphogenic modulator is vital for all plant processes throughout their life cycle.

The most recent studies suggest that the circadian regulatory system is comprised of three main components: time-giving input pathways mediated by environmental stimuli; a central oscillator that generates the circadian rhythm; and output pathways that influence a range of physiological processes (Sharma *et al.,* 2022). The most important external cue for the clock is light, as it is a complex environmental signal that changes in intensity, daylength and quality. Plant light receptors, each with specific wavelength absorptions (e.g., phytochromes, cryptochromes, Zeitlupe family, UV RESISTANCE LOCUS 8), provide irradiance and spectrum based signalling to the clock allowing the plant to perceive time of day or season (Oakenfull and Greenham, 2017, Oravec and Greenham, 2022). Red and far-red light are primarily perceived by phytochromes (phyA-phyE), whereas blue light is detected by cryptochromes (CRY1 and CRY2) and members of the Zeitlupe family (Oakenfull and Greenham, 2017, Oravec and Greenham, 2022). UV-B radiation is sensed by UVR8. The input from these timing-givers regulates the expression of core oscillators within the clock (Xu *et al.,* 2022). The central oscillator consists of a series of interconnected transcriptional-translational feedback loops that generate the self-sustained oscillations (Linde *et al.,* 2017). These feedback loops provide robustness and flexibility, allowing the clock to remain synchronised with changing environmental stimuli.

The main feedback loop is comprised of morning-phased transcription factors, *CIRCADIAN CLOCK ASSOCIATED (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION (TOC1)* and additional *PSEUDO-RESPONSE REGULATORs (PRRs)* (Gil and Park, 2018). The evening complex involves a myeloblastosis transcription factor *LUX ARRHYTHMO (LUX)* and two transcriptional regulators, *EARLY FLOWERING 3 (ELF3)* and *ELF4* (Gil and Park, 2018). Within the feedback loop, both *CCA1* and *LHY* repress *TOC1* and *GIGANTEA (GI)* expression in the morning, whereas in the evening *TOC1* is the repressor of *CCA1* and *LHY* (Gendron *et al.,* 2012). Furthermore, *PRR* genes such as *PRR5*, *PRR7*, *PRR9* and *TOC1* are expressed sequentially throughout the day, which progressively repress *CCA1* and *LHY*, ensuring accurate timing of morning and evening gene expression (Nakamichi *et al.,* 2009). Rhythmic activity generated by the central oscillator is transmitted through numerous output pathways that regulate diverse physiological processes. Of particular relevance to this study the circadian clock regulates the expression of *CYCLIN DOF FACTOR* (*CDF*) genes, which integrate circadian timing with photoperiod flowering (Praena *et al.,* 2022).

In addition to regulating plant growth and developmental processes, the circadian clock is critical for the modulation of plant responses to abiotic stress. Studies that have altered expression of clock components, showed alterations in sensitivity to specific abiotic stresses such as salinity and drought, highlight a clear link between the circadian clock and stress responses (Seo and Mas, 2015). For example the over expression of circadian regulated *RESPONSE TO DESSICATION* (*RD29A*) and *SALT OVERLY SENSITIVE* 1 (*SOS1*) showed improved salinity tolerance in *Arabidopsis thaliana* and *Solanum lycopersicum* (Park *et al.,* 2016, Kaur *et al.,* 2025). Changes in the expression of *PRR5*, *PRR7* and *PRR9* show improved tolerance to drought stress, similarly the reduced expression of *TOC1* saw increased drought tolerance in *A. thaliana* (Grundy *et al.,* 2015). Interestingly *PRRs* work downstream on *CYCLIN DOF FACTOR 1* (*CDF1*), which is also associated with improved drought tolerance in *A. thaliana* (Nakamichi *et al.,* 2007, Blair *et al.,* 2022).

1.2 Drought Stress and Flowering Time Regulation in *Arabidopsis thaliana*

Flowering is a crucial step in a plant's life cycle to ensure seed production for future generations to secure the survival of species. To maximise reproductive outcomes, plants have adapted to

initiate flowering during favourable environmental conditions. This transition from vegetative to reproductive growth is regulated by a complex network of genetic pathways that respond to endogenous and environmental stimuli (Kazan and Lyons, 2015). These pathways include: age, vernalization, photoperiod, gibberellic acid *(GA)*, autonomous and circadian clock, all converging to regulate the floral pathway integrator genes (Maple *et al.,* 2024). The floral transition is initiated by the accumulation of florigen in response to favourable environmental conditions. In Arabidopsis the floral pathway integrator FLOWERING LOCUS T (FT) is the main florigen, the expression of which is activated by the photoperiod-dependent transcription factor CONSTANS (CO) under long day conditions (Martingnago *et al.,* 2023, Figure 1). FT is synthesized in the leaves and transported to the shoot apical meristem, where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) to activate the floral pathway integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), as well as floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Lee *et al.,* 2023). *SOC1* encodes a MADS-box transcription factor that helps to convert environmental signals into a developmental response (Lee and Lee, 2010). This interaction thereby initiates the transition from vegetative to reproductive growth. The B-box transcription factor CO is regulated by the circadian clock at the transcriptional and post-translational level, while studies also show that *CO* and *FT* expression is repressed throughout the day by CDFs as they directly bind to the promoter regions (Praena *et al.,* 2022). However, as these floral pathways are influenced by the environment any stress stimuli can alter growth and development involving a range of molecular responses which will be addressed in detail later in this section.

As the climate continues to change, with increased shortages of freshwater resources, drought is now the most common abiotic stress around the world (Chen *et al.,* 2023). Drought-induced acceleration of flowering, referred to as the drought escape response occurs primarily under mild to moderate drought conditions and relies on hormonal signalling, circadian clock components and stress-responsive transcriptional networks (Takeno, 2016, Figure 1). Under long day (LD) conditions, *Arabidopsis* displays an early flowering phenotype in response to drought stress, to ensure seed production before the stress becomes lethal (Cho *et al.,* 2016). This early flowering is dependent on the function of the flowering gene *GIGANTEA* (*GI*) and *SOC1* combined with the upregulation of *FT* (Chen *et al.,* 2023, Figure 1). Under drought conditions the degradation of CDF1 is triggered by GI, FLAVIN-BINDING, KELCH REPEAT AND F BOX protein 1 (FKF), promoting the upregulation of *CO*, which in turn promotes flowering (Tun *et al.,* 2021). CDF1 acts downstream of circadian cues to repress *CO*,

creating an additional layer of regulation to fine-tune flowering time (Corrales *et al.,* 2017). Interestingly, *CDF1* and its antisense transcript *lncCDF1* respond to abiotic stress, suggesting an important role in integrating drought and circadian signalling pathways to modulate stress responses in *A. thaliana* (Figure 5).

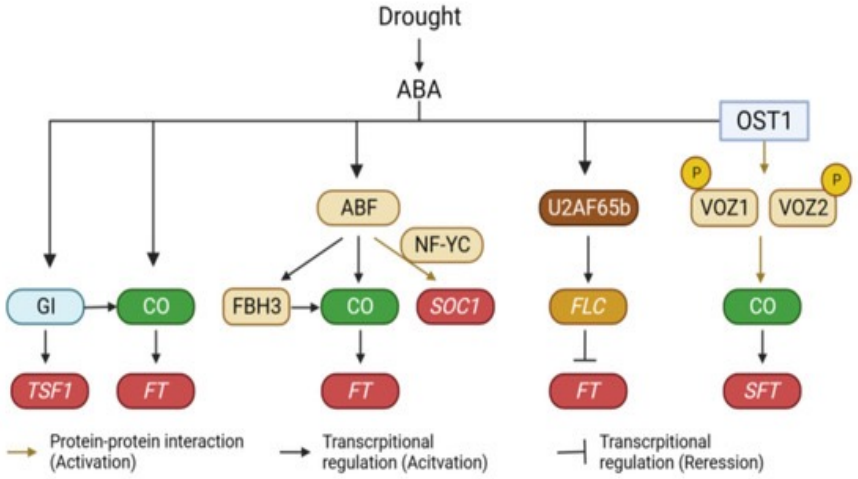


Figure 1: Flowering time regulation under drought conditions. Drought serves as a positive environmental cue that promotes flowering in plants by influencing multiple components of the flowering time regulatory pathway. GIGANTEA (GI), CONSTANS (CO), TWIN SISTER OF FT (TSF1), FLOWERING LOCUS T (FT), ABA-RESPONSIVE ELEMENT BINDING FACTOR (ABRE), NUCLEAR FACTOR Y SUBUNIT C (NF-YC), FLOWERING BHLH 3 (FBH3), SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), FLOWERING LOCUS C (FLC), OPEN STOMATA 1 (OST1), VASCULAR PLANT ONE-ZINC FINGER 1 (VOZ1) (Lee *et al.,* 2023) .

1.3 Long non-coding RNAs as Regulators of Biological Processes in Plants

Long non-coding RNAs (lncRNAs) are a diverse class of transcripts that lack a protein coding ability and have a length greater than 200 nucleotides. However, recent studies highlight their significance in the regulation of gene expression in eukaryotes, affecting a range of molecular processes from transcription, mRNA stability, post-translational modifications and chromatin architecture (Budak *et al.,* 2020). Similar to mRNAs, the majority of lncRNAs are transcribed by RNA polymerase II, but in plants a small fraction of lncRNAs are transcribed by plant-specific RNA polymerases IV and V (Wu *et al.,* 2020). There are multiple types of lncRNAs which are classified by their influence on targeted DNA sequences, biogenesis and functional

mechanism, these include: long intergenic lncRNA (lincRNA), intronic lncRNA (incRNA), sense lncRNA and natural antisense transcripts (NATs) (Wu *et al.,* 2020). These transcripts are similar to mRNAs that undergo 5'-end capping, the addition of a 3' ploy-A tail, and are subject to alternative splicing, although some lncRNAs are non-polyadenylated at the 3' end (Lu *et al.,* 2017, Budak *et al.,* 2020). Unlike mRNAs, lncRNAs display stage and tissue-specific expression at low levels, while being poorly conserved across species (Lu *et al.,* 2017). They interact with DNA, RNA and proteins, while functioning either *in cis* or *in trans* manner (Jampala *et al.,* 2021).

With the rise of lncRNAs as key regulators in major plant processes, studies began exploring their potential in abiotic stress responses. lncRNAs tend to be selectively activated in response to specific stress stimuli, indicating biological importance for stress adaption (Di *et al.,* 2014). Studies suggest that lncRNAs can target stress responsive genes and positively regulate their expression allowing plants to be more tolerant to stress (Tun *et al.,* 2023). For example, *DROUGHT INDUCED lncRNA* (*DRIR*) is a lincRNA that is localized in the nucleus and controls several drought responsive genes (Jha *et al.,* 2020). Similarly, lncRNAs are reported to regulate multiple drought stress regulatory genes involved in starch and soluble sugar synthesis, and ABA synthesis and signalling (Jha *et al.,* 2020). Nonetheless, few have been characterised to regulating plant responses to abiotic stress.

Within the different *A. thaliana* lncRNAs, there are ~30,000 *NATs* and over 70% of its protein-coding loci have encoding potential for NAT pairs (Chekanova, 2015). NATs are RNA molecules that are transcribed from the opposite DNA strand of protein-coding genes (Werner *et al.,* 2024). These NAT pairs can regulate important biological functions. For instance, the gene *UGT73C6* has two lncNATs, $NAT1_{UGT73C6}$ *and* $NAT2_{UGT73C6'}$, and their overexpression leads to increased rosette size, suggesting a function in plant growth regulation (Meena *et al.,* 2021). Furthermore the NAT pair *lncCDF5/CDF5* can optimise its circadian oscillations to regulate photoperiodic flowering (Henriques *et al.,* 2017, Figure 2). Given that *lncCDF5* can function *in trans* to modulate other *CDF* genes *(CDF1,CDF3*), we sought to determine whether other *CDF* associated NAT pairs, such as *lncCDF1/CDF1*, play similar regulatory roles.

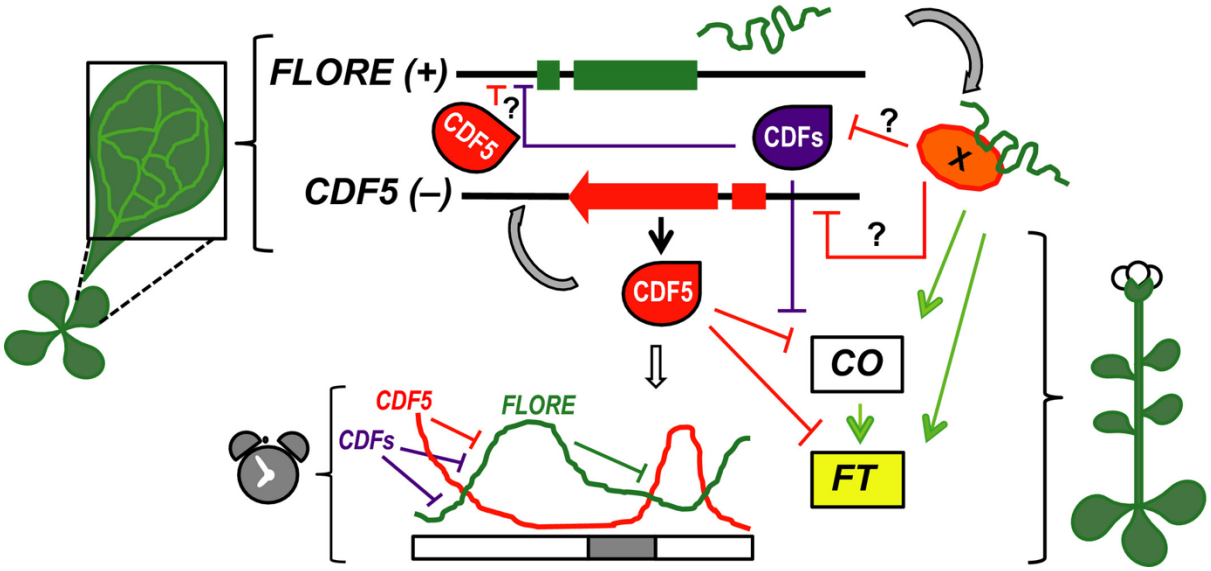


Figure 2: Proposed model of the regulatory relationship between *CYCLING DOF FACTOR 5* (*CDF5*) and *CDF5 long non-coding RNA* (*FLORE*) in *A. thaliana*. *CDF5* and *FLORE* form a circadian-regulated natural antisense transcript pair with antiphasic expression. Their rhythmic expression is maintained through reciprocal repression, whereby *CDF5* suppresses *FLORE* accumulation during the afternoon, while *FLORE* negatively regulates *CDF5* expression in the morning. Other members of the *CDF* family including *CDF1*, *CDF2* and *CDF3*, contribute to the repression of *FLORE* (purple lines). *CDF5* and *FLORE* accumulate in the vascular tissue where they oppositely regulate the *CONSTANS* (*CO*)-*FLOWERING LOCUS T* (*FT*) module. Straight end lines indicate repression, while green arrows represent transcriptional activation. The circadian expression profiles of *CDF5* (red) and *FLORE* (green) are shown, where the grey rectangle indicates the dark phase during long-day photoperiods. Unresolved regulatory mechanisms are denoted by (?) (Henriques *et al*., 2017).

1.4 The Role of *CDFs* in Circadian and Stress Signalling

CDFs are a subgroup of DNA BINDING WITH ONE FINGER (DOF) proteins. DOF genes are classified into four major clusters (A, B, C and D), with a total of 37 DOF genes identified in *A. thaliana* (Qin *et al.,* 2023). Group D consists of DOF factors whose transcript continue to oscillate under constant light conditions, known as CDFs (CDF1-5) (Imaizumi *et al.,* 2005). These DOF proteins are plant specific transcription factors with a DNA binding domain capable of regulating gene expression and abiotic stress signalling, with reports suggesting a wider range of functions such as nuclear localization, cell-to-cell trafficking and interacting with other transcription factors (Renau-Morata *et al.,* 2020). This DNA binding domain is predominantly located near the N-terminal region of the protein and is a conserved region of 52 amino acid residues (Corrales *et al.,* 2017).

In *Arabidopsis*, CDFs have linked the circadian clock to the photoperiodic flowering pathway as mentioned, through the direct binding of *CO* and *FT* promoters (Henriques *et al.,* 2017). The CDF family members exhibit functional redundancy, collectively acting as transcriptional repressors of photoperiodic flowering genes, where mutations of a single CDF gene may not produce a strong phenotype (Fornara *et al.,* 2009). The oscillations of these CDF transcription factors are regulated by multiple circadian clock components (Blair *et al.,* 2022). CDFs generally accumulate early in the morning and throughout the day they are slowly degraded (Fornara *et al.,* 2009). *CDF5* and its antisense transcript undergo regulation by *CCA1*, *PRR9*, *PRR7* and *PRR*5, while *CDF3* is associated with *PRR7* and *PRR5* (Henriques *et al.,* 2017, Nakamachi *et al.,* 2012). In particular *CDF1* is negatively regulated by *PRR9*, *PRR7* and *PRR5*, which repress *CDF1* mRNA accumulation, but is positively regulated by *CCA1* via regulation of *GI* (Nakamichi *et al.,* 2007). *CDF1* is additionally regulated at the transcriptional and post transcriptional level by *lncCDF1*, which contributes to the fine-tuning of *CDF1* expression under circadian control (Figure 3). Moreover, *CDF1* and *CDF5* accumulation can promote hypocotyl elongation in *A. thaliana* seedlings, while under photoperiodic regulation (Martín *et al.,* 2020). As well as having key functions in biological processes such as plant development, CDFs have been described to also function in response to abiotic stress (Renau-Morata *et al.,* 2020). However few studies have elucidated the relationship between CDFs and their NAT counterparts in abiotic stress responses.

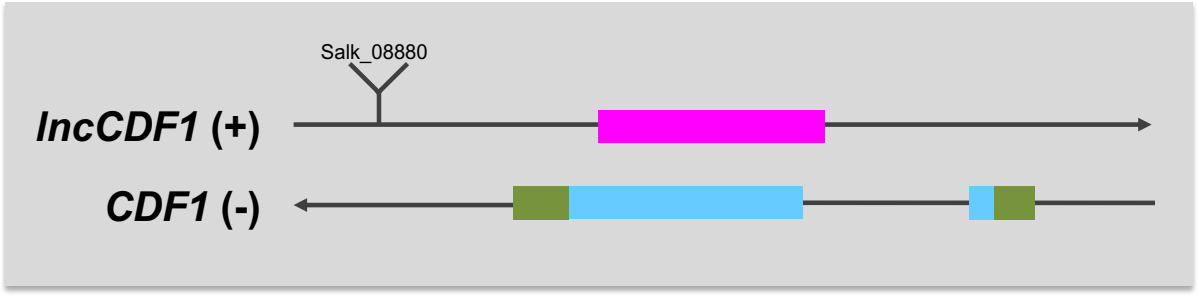


Figure 3: Schematics provided by Dr. Henriques of the *CDF1/lncCDF1* locus. The green rectangles depict 5'UnTranslated Region and 3'UnTranslated Region, and the black lines are introns. The light blue rectangles are *CDF1* exons and the pink rectangles are *lncCDF1* exons. (+) and (-) represent sense and antisense strands respectively. (Salk_08880) indicates the T-DNA insertion in the promoter region of *lncCDF1* for the *lnccdf1 Salk-mutant #08880-10.*

As mentioned above, beyond their role in circadian and photoperiodic regulation, CDFs are recognised as contributors to abiotic stress responses, functioning as integrators of

environmental signals (Renau-Morata *et al.,* 2020). They act indirectly on abiotic stress tolerance by activating or repressing key stress responsive genes (Waqas *et al.,* 2020). *CDF3* overexpression enhances plant tolerance to abiotic stress such as cold, salinity and drought by regulating the *CBF/DREB* and *ZAT10/12* pathways (Jin *et al.,* 2024). In potato, *StCDF1* in conjunction with its antisense lncRNA *StFLORE* modulates water loss by altering stomatal development and its diurnal opening (Kloosterman *et al.,* 2013). *CDF1* has previously been linked to abiotic stress in *Arabidopsis*, but few studies have characterised its effect on drought stress responses, in particular the regulatory role of the NAT pair *lncCDF1/CDF1*.

1.5 Carbohydrate Metabolism Under Drought Stress

Sugar metabolism is a fundamental process in plant growth, development and stress adaption, as it maintains an energy source and provides structural components. The circadian clock plays a central role in co-ordinating sugar metabolism with daily light-dark cycles, ensuring carbohydrate synthesis, storage and remobilization are synchronised with energy demand and environmental conditions (Venkat and Muneer, 2022). Sugars such as glucose and sucrose are involved in the regulation of major cellular processes in plants (Baier *et al.,* 2003). Glucose has been reported to transcriptionally regulate 62% of the genes affect by indole-3-acetic acid (IAA), where increased glucose concentration promoted lateral root development (Mishra *et al.,* 2009). Furthermore, glucose and sucrose form part of a feed-forward loop that controls the conversion of sucrose to glucose via Cytosolic invertase 1 (CINV1) which in turn controls the juvenile-to-adult phase transition in *A. thaliana* (Meng *et al.,* 2021). Sucrose is the most abundant transported sugar, accounting for over 80% of total transported solute in the phloem (Liu *et al.,* 2012). Starch is one of the primary products of photosynthesis and serves as a storage unit to withstand changes in photosynthate availability due to light and dark cycles (Streb and Zeeman, 2012). In *A. thaliana* glucose, fructose and sucrose are synthesized throughout the day, with a portion converted to starch that can be remobilized at night (Bläsing *et al.,* 2005). This starch is used as a continuous carbohydrate source when photosynthesis is halted. The circadian clock is responsible for regulating the transcript amounts of multiple enzymes associated with primary metabolism in plants and under normal growth conditions, 30% of metabolite accumulation is controlled by the clock (Kim *et al.,* 2017).

Sugars form part of a regulatory network with light and circadian mediated signalling pathways, to integrate carbohydrate synthesis and use in response to environmental changes (Baier *et al.,* 2003). This network modulates the expression of sugar responsive genes which are involved in resource allocation and cellular adjustment to nutrient availability (Kunz *et al.,* 2014). The type or nature of a sugar is significant with regards to metabolic response, but its concentration also plays an important role. For example, sugars will induce the growth of roots, until it reaches an internal concentration close to 3% where its effect decreases (Pereyra *et al.,* 2023). Carbohydrate accumulation and allocation is vital for ensuring plasticity to tolerate harsh growing conditions. For example, in response to cold or drought plants can compromise growth over photosynthesis to accommodate the accumulation of greater sugar stores throughout a mild stress (Pereyra *et al.,* 2023). Recent studies suggest that the lncRNA, *COLD INDUCED lncRNA 1* (*CIL1*), acts as a positive regulator of cold stress responses in *A. thaliana*, where induction of this lncRNA altered the expression of multiple genes involved in glucose metabolism (Liu *et al.,* 2022). This finding highlights the potential for lncRNAs to act as important regulators of sugar metabolism and stress responses.

Drought stress causes the plant to reset its source-sink relationship, which in turn disrupts cellular processes and sugar transport (Kaur *et al.,* 2021). Sugar accumulation during drought stress has been well described as an important process for coping with drought stress. For example, drought exposed *A. thaliana* had a higher contribution from soluble sugars rather than proline in cellular osmotic adjustment (Gurrieri *et al.,* 2020). Moreover, drought stress also promotes the breakdown of starch into soluble sugars, decreasing the water potential of the cell to enhance water uptake without changing total non-structural carbohydrates (Camisón *et al.,* 2020). Studies show that the orphan gene *QUA QUINE STARCH (QQS)* plays a role in regulating starch accumulation by inhibiting carbon partitioning into starch biosynthesis (O'Conner *et al.,* 2018). The overexpression of *QQS* in *A. thaliana* leads to a decrease in starch accumulation and an increase in protein accumulation (O'Conner *et al.,* 2018). The transport of soluble sugars from source to sink organs is facilitated by *SUCROSE TRANSPORT GENES (SUC)*, capable of transporting a wide range of substrates (Bavnhøj *et al.,* 2023). Previous studies suggest a role for NAT pairs as an underlying mechanism to fine-tune the regulation of key sugar related genes under drought conditions. However, the specific contribution of lncRNAs and their sense counterparts remains largely unexplored.

1.6 Research Objectives

This study aims to elucidate the role the of *lncCDF1* and *CDF1* in mediating drought stress responses and flowering time regulation in *A. thaliana*. Specifically, this research project included the following objectives:

1. To determine how different levels of *lncCDF1* and *CDF1* impact flowering time regulation under control and drought conditions. In order to assess this I tested Col-0 (wildtype, WT), the *lnccdf1* knockdown mutant (*lnccdf1 Salk-mutant #08880-10)*, overexpressor lines *pSUC2::lncCDF1 #1.3* and *#2.3*, the *cdf* quadruple mutant (*cdf-q;* Fornara *et al.,* 2009) and the *CDF1* overexpressor line (*HA-CDF1*; Imaizumi *et al*., 2005), to determine how this NAT pair influences the coordination between stress perception and developmental transitions.
2. To validate, using real time quantitative RT-PCR, the differentially expressed genes identified by RNA-seq analysis of *lnccdf1* mutants grown under control and drought conditions. To accomplish this, I determined the expression levels of *QUA QUINE STARCH (QQS)*, *SUCROSE-PROTON SYMPORTER 7 (SUC7)*, *OSMOTIN 34 (OSM34)*, *EXTENSIN 1 (EXT1)*, and *MOTHER OF FT AND TFL (MFT)* by RT-qPCR.
3. To evaluate the starch and soluble sugar levels of *Arabidopsis* lines accumulating different levels of *lncCDF1* and *CDF1* (described in 1.) grown under control and drought conditions. To achieve this, I determined the starch and soluble sugar (glucose, fructose, sucrose) contents of Col-0 (wildtype, WT), *lnccdf1* knockdown mutant (*lnccdf1 Salk-mutant #08880-10*), overexpressor lines *pSUC2::lncCDF1 #1.3* and *#2.3,* and *CDF1* overexpressor line (*HA-CDF1*; Imaizumi *et al*., 2005).

In summary, this research projects aims to establish a link between the *lncCDF1/CDF1* NAT pair and the regulation of drought responsive genes, the time to flower and the accumulation of soluble and insoluble sugars. These findings will provide a baseline of how *lncCDF1/CDF1* may integrate stress responses with metabolic and developmental processes in *A. thaliana*.

2.0 Materials and Methods

2.1 Assessment of Flowering Time

The following procedures were adapted from Praena *et al.,* (2022)

2.1.1 Synchronisation and Germination of seeds

Plant materials: *Arabidopsis thaliana* Col-0 seeds were used as wildtype control.

*pSUC2::lncCDF*1 *#1.3* and *#2.3* seeds: *pSUC2::lncCDF*1 *#1.3* and *#2.3* are transgenic overexpression lines engineered to express *lncCDF1* transcripts under the control of the *SUCROSE-PROTON SYMPORTER 2* promoter (*pSUC2*). The two independent transgenic lines differ in their level of *lncCDF1* overexpression, with line #1.3 exhibiting stronger expression than line #2.3, approximately five-fold higher expression (Dr. Henriques).

lnccdf1 Salk-mutant #08880-10 seeds: The *lnccdf1 mutant* is a knock down mutant with a T-DNA insertion upstream of the promoter region of the *lncCDF1* locus.

cdf-q seeds: The *cdf-quadruple* mutant is a quadruple loss-of-function *Arabidopsis* line where four *CDFs* (*CDF1, CDF2, CDF3* and *CDF5*) have been simultaneously knocked out (Fornara *et al.,* 2009).

HA-CDF1 seeds: The *HA-CDF1* line is a transgenic line expressing the CDF1 protein fused to a hemagglutinin (HA) epitope tag. This line is driven by a strong constitutive promoter *35s::HA-CDF1* which overexpresses HA-tagged *CDF1* (Imaizumi *et al*., 2005).

This procedure used seeds previously harvested in the lab.

Procedure

First I placed the soil plugs in the plastic tray, one plug for each genotype. Then I added ~400 ml of water to the tray with each plug individually labelled. Arabidopsis seeds of each tested genotype were then dispersed on each plug to ensure 10-15 seeds were sown. To synchronise germination, we placed a second plastic tray over the plugs creating a dome, then wrapped completely with aluminium foil. Seeds were placed in a 4°C fridge for 3-7 days for stratification. Following the stratification period, all aluminium foil was removed and the trays

of seeds were placed in a growth chamber under long-day conditions at 22°C to allow the seeds to germinate. Once the seeds had germinated I ensured to water every 2-3 days until the seedlings reach ~1 cm, where I then separated seedlings into individual soil plugs (Figure 4A). Seedlings were kept well-watered until the drought treatment. To collect seeds from the dried *A. thaliana* plant material, we lightly agitated the plant over the coarse sieve and discarded the stems and petals, ensuring all seed capsules have opened to obtain all the seeds. This step was repeated with both the fine and coarse sieve on top of each other. The seeds were collected and stored in their respective Eppendorf tubes.

2.1.2 Thinning of Plant Material and Drought Treatment

Materials: Seedlings prepared from the previous method (2.1.1), *Jiffy* soil plugs, plastic tray, forceps, plastic cups, waterproof tape.

Procedure

Sixteen soil plugs were prepared for each genotype. Once seedlings reached 1cm in height they were thinned. Using forceps, a small hole was made in each new plug for transplantation. A seedling was then carefully selected with a forceps and transplanted into the new plug (Figure 4B). The seedling was secured in the new plug by gently pressing down the soil around it. Seedlings were subsequently placed into a long-day growth chamber at 22°C, and allowed to develop for 3-4 days before the drought treatment was applied.

Before the drought treatment, all plants were ensured to be well watered. A plastic cup was prepared for each plant by cutting a hole for the plant at the bottom of the cup to allow the seedling to pass through. The cup was then cut to fit the size of the soil plug. This cup was then placed over the soil plug leaving just the seedling exposed. Waterproof tape was used to seal the bottom, this was to ensure all water loss is through the plant (Figure 4C). Each soil plug was weighed and the weight recorded was taken as 100% water content. Plants were maintained at 100% water content for 6-7 days. Once all plants had developed rosettes, drought stress was induced by reducing watering to 50% water content. Control plants were kept at 100% water content for the duration of the experiment. The drought treatment was maintained until sample collection.

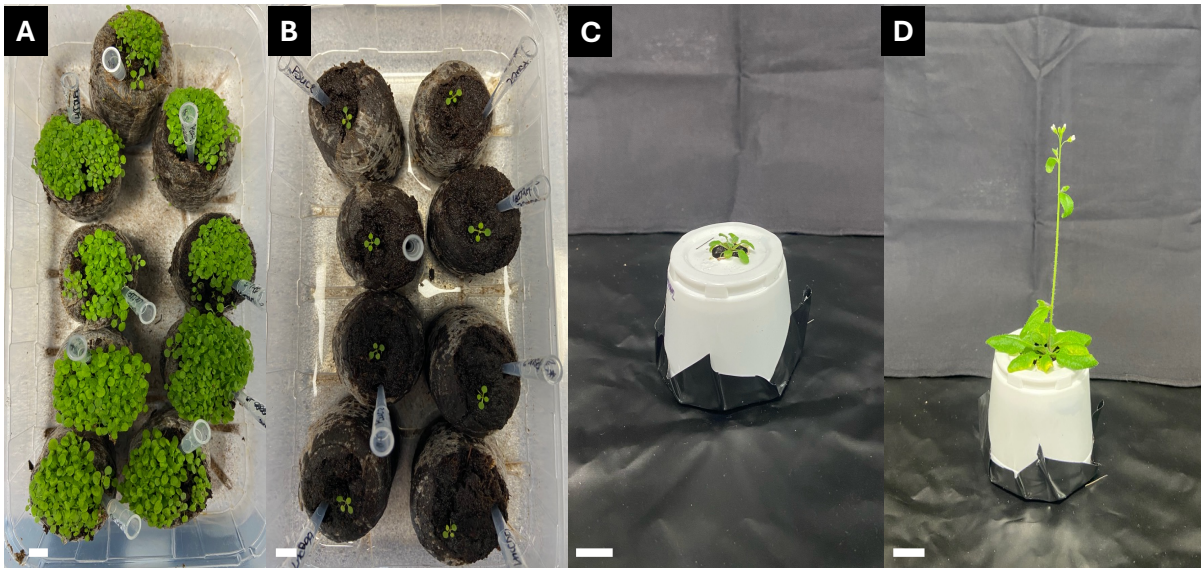


Figure 4: (A) *A. thaliana* seedlings in soil plugs; (B) Transplanted individual seedlings; (C) Sealed soil plug for drought treatment; (D) Developing plant under drought conditions. All scale bars equal to 1cm

2.1.3 Scoring of Flowering Time

For this study flowering time was recorded as the days until bolting occurs. Bolting was measured when the main inflorescence reached 1cm. Days to flowering were counted starting from when seeds were placed in the light. Flowering time was also measured by recording leaf number. Both rosette and cauline leaves were recorded.

Procedure

For the first 2 weeks of development, a day in the week was assigned to record leaf number. On the first day a marker was used to score each leaf with a dot and rosette leaf number was recorded on a table. We continued to score rosette leaves until bolting occurred (~3 weeks). Once bolting occurred plants were monitored every 2 days to ensure accurate recordings of flowering time. As the plant transitions to flower cauline leaves were recorded.

2.2 Gene Expression Analysis

For the purpose of this study, gene expression analysis was conducted at three Zeitgeber Time (ZT) sampling points: ZT1.5, ZT6 and ZT9, where ZT0 corresponds to lights-on and subsequent ZT values indicate hours after the beginning of the light phase.

2.2.1 RNA Extraction

Total RNA was extracted from *A. thaliana* rosette tissue using the Qiagen RNeasy Plant Mini Kit following the manufacturer's instructions, including the preparation of DNase stock solution. 10 µl of DNase were then mixed with 70 µl of RDD per sample. RLT buffer was also prepared by adding 10 µl β-Mercaptoethanol per 1 ml RLT buffer. Rosette tissue was collected from 2 plants per sample, combined and frozen using liquid nitrogen to be stored at -80°C

Materials: 100% ethanol, DNase, RDD buffer, RLT buffer, RPE buffer, β-Mercaptoethanol, RNA-free water

Procedure

Briefly, 450 µl of RLT were added to ~100 mg of tissue powder, transferred to the shredder column and centrifuged at full speed for 2 minutes. The flow through was then mixed with 50% the total volume of 100% ethanol (225 µl) and transferred to the RNA binding column and centrifuged 10,000 rpm for 1 minute. This column was then washed with 350 µl of RW1 buffer and centrifuged 10,000 rpm for 1 minute. 80 µl of DNase mix were directly added to the column membrane and incubated at room temperature for 15 minutes. This was then washed with 350 µl RW1 buffer and centrifuged at 10,000 rpm for 1 minute. Then 500 µl of RPE buffer was added and tubes were centrifuged at 10,000 rpm for 30 seconds. This washing step was repeated and the membrane was then dried by centrifugation. The column was placed in a new Eppendorf tube to which 50 µl of RNAse-free water was added and centrifuged at 10,000 rpm for 1 minute. This step was repeated using the same eluate to maximise RNA concentration which was then assessed using a NanoDrop spectrophotometer. RNA samples were then stored at -20°C for future use.

2.2.2 *Oligo dT* cDNA Synthesis

cDNA was synthesized using the Agilent AffinityScript cDNA Synthesis Kit. For genotypes Col-0 and *lnccdf1 Salk-mutant #08880-10* first strand cDNA was synthesized from 1 µg of total RNA. For genotypes Col-0, *pSUC2::lncCDF*1 *#1.3* and *#2.3*, and *HA-CDF1* cDNA was synthesized using 0.5 µg of total RNA. 0.5 µg of total RNA was used for these genotypes as the RNA quantity extracted was not enough for 1 µg. Col-0 was repeated as a control

Procedure

RNA was prepared using 1µg in volume up to 6 µl RNase-free dH_20. The reaction mix contained 10 µl 2x Master Mix, 3 µl *oligo dT* primer (100 ng/µl) and 1 µl *AffinityScript RT/RNase block* for final volume of 20 µl. The reaction was gently mixed, then incubated at 25°C for 2 minutes to allow primer annealing. Followed by 42°C for 50 minutes to synthesize cDNA. Lastly 95°C for 5 minutes to inactivate the reverse transcriptase. cDNA was diluted 1:20 with dH_2O to a final volume of 40 µl. The procedure was repeated for 0.5 µg total *RNA* samples.

2.2.3 Strand Specific cDNA Synthesis

cDNA was synthesized using the *Agilent AffinityScript cDNA Synthesis Kit.* For genotypes Col-0 and *lnccdf1 Salk-mutant #08880-10* strand specific cDNA was synthesized from 1 µg of total RNA. For genotypes Col-0, *pSUC2::lncCDF*1 *#1.3* and *#2.3*, and *HA-CDF1* cDNA was synthesized using 0.5 µg of total RNA.

Procedure

RNA was prepared using 1 µg in volume up to 6 µl RNase-free dH_2O. The reaction mix contained 10 µl 2x Master Mix, 1.5 µl *lncCDF1 TOPO-Rev* (+) p1 (~200 ng/µl), 1.5 µl *actin-2* q160-R (~200 ng/µl) and 1 µl *AffinityScript RT/RNase block* for final volume of 20 µl (Table 1). The reaction was gently mixed, then incubated as in the previous procedure. Strand specific cDNA was diluted 1:20 with dH_2O to a final volume of 40 µl. The procedure was repeated for 0.5 µg total RNA samples.

Table 1: Primers used for *lncCDF1* strand specific cDNA.

Amplied product	Primer name	Sequence	Reference
lncCDF1	lncCDF1-TOPO-Rev	TTTCTTTATTCATCATCCGTCCCTAATAATCC	Dr. Henriques
	actin2-q160-R	AGAAACCCTCGTAGATTGGCACAGT	

2.2.4 Quantitative Real-Time RT-PCR (qRT-PCR) Analysis

qPCR was performed to quantify transcript levels of *CDF1*, *lncCDF1*, *QQS*, *SUC7*, *OSM34*, *MFT* and *EXT1*. This protocol uses the *SYBR ExTaq (TAKARA) mi*x. *Actin* was used as a

reference gene for all reactions. Strand specific cDNA was used for *lncCDF1* reactions, while oligo dT cDNA was used for all other genes.

Procedure

For cDNA prepared using 1 µg of RNA the reaction mix contained 5 µl SYBR Ex-Taq mix, 0.2 µl forward primer (10 µM), 0.2 µl reverse primer (10 µM), 0.2 µl ROX Reference Dye II, 1 µl cDNA and 3.4 µl dH_2O, for a final volume of 10 µl. For cDNA prepared using 0.5 µg of RNA the reaction mix used 2 µl cDNA and dH_2O was adjusted to make a final volume of 10 µl (Table 2). Each sample was run in technical triplicates. Thermal cycling was performed with the following program 94°C for 30 seconds, then amplified for 40 cycles of 94°C for 10 seconds followed by 60°C for 30 seconds. After amplification a melt curve determination was performed (94°C for 15 seconds, the gradually increase from 60°C to 94°C with 0.5°C increments for 15 seconds)

Gene expression was calculating using the $2^{-\Delta\Delta Ct}$ method and results were normalized using Actin. For all reactions Col-0 ZT6 expression value was used as reference (value of 1).

Table 2: Primers used for quantitative real-time RT-qPCR.

Amplified product	Primer name	Sequence	Reference
lncCDF1	lncCDF1-q1F	GCGAGGTTGGTTTACGTTGTAGTTGT	Dr. Henriques
	lncCDF1-q1R	TTGCATCAGAACCAAAACAAGACATTA	
CDF1	CDF1-q1F	AAGAAGAAGAAAAGAACCAAAACAAGACATTA	Henriques *et al.* , 2017
	CDF1-q1R	CACCTGAGGTCCAATATCTCTGACAAG	
Actin2	actin2-q160F	AAGGCCAACAGAGAGAAGATGACTCA	Henriques *et al.* , 2017
	actin2-q160R	AGAAACCCTCGTAGATTGGCACAGT	
QQS	QQS-q2F	ACGTTGAAAGAAGCTTCAAACCAAA	Kyun's lab, Seoul National University, Republic of Korea
	QQs-q2R	GCCTTGCGACACCTGATGTA	
SUC7	SUC7-q1F	ACAACAAACACAACCTCTAA	Dingenen *et al.* , 2017
	SUC7-q1R	CTATCCACAGTCGTCTCA	
OSM34	OSM34-q1F	CTCTCAACACGTTTGGACATTGTC	Park and Kim, 2021
	OSM34-q1R	TTGAACCAATTCAACAACTTAGAC	
MFT	MFT-q1F	ATCACTAACGGCTGCGAGAT	Barros-Galvao *et al* ., 2019
	MFT-q1R	CGGGAATATCCACGACAATC	
EXT1	EXT1-q1F	ACAAATCACCACCTCCTCCTCACT	Saha *et al.* , 2013
	EXT1-q1R	TCTCCCGTCAACGATCTTGTGTCT	

2.3 Quantification of Starch and Soluble Sugars

In this study, sample timepoints ZT1.5, ZT6, ZT9 and ZT16 were used for quantification.

2.3.1 Extraction of Starch and Soluble Sugars

Starch and sugar extractions were performed on genotypes: Col-0, *lnccdf1 Salk-mutant #08880-10, pSUC2::lncCDF*1 *#1.3* and *#2.3*, and *HA-CDF1,* both control and drought. Frozen *A. thaliana* rosette tissue powder from fully developed plants (~ 3 weeks old) was used for extraction. Each sample contained the rosette tissue of 2 plants.

Materials: 80% ethanol, 0.5 M sodium hydroxide (NaOH), 0.5 M hydrochloric acid (HCl), 0.2 M sodium acetate (NaOAc, pH5.2) containing 1 unit of amyloglucosidase from *Aspergillus niger* per sample, RNAse-free water.

Protocol

First 2 Eppendorf tubes were prepared and labelled “Starch” and “Sugar”, and only to the “Starch” tube was 50 mg of sample added. Then 1 ml of 80% ethanol was added to the sample, which gets vortexed and incubates at 80°C for 30 minutes, followed by 5 minute centrifuge at max speed. The supernatant was then transferred to the “Sugar” Eppendorf and another 1ml of 80% ethanol was added to the “Starch” Eppendorf to repeat the previous step. Both extracts were combined in the “Sugar” Eppendorf for a total volume of 2 ml. The “Sugar” Eppendorf with the ethanol extract was dried in a speed vac overnight. The dried extract then gets dissolved in 10 µl of miliQ water per mg Fresh Weight and centrifuged at full speed for 5 minutes. The supernatant was used for sugar determination. The “Starch” Eppendorf containing the pellet was incubated overnight at 37°C with the lids open to dry residual ethanol . Once the pellet was dry, 200 µl of 0.5 M NaOH was added to the sample, which was vortexed and incubated for 60 minutes at 60°C. After incubation 200 µl of 0.5 M HCL was added and the sample was vortexed again. Finally 600 µl of 0.5 M NaOAc (pH:5.2) containing 1 unit of amyloglucosidase per sample was added, and was then gently mixed and incubated at 37°C overnight. Following incubation we sealed the Eppendorf lids with Parafilm and heated to 100°C for 2 minutes to inactivate the enzyme. A final centrifuge at full speed for 5 minutes was carried out before the supernatant was used for starch determination.

2.3.2 Determination of Starch and Soluble Sugars

To determine starch content in *A. thaliana* samples, the starch was converted to glucose using the previous procedure.

Materials: HEPES buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/5 mM magnesium chloride($MgCl_2$), pH 7.5), 40 mM nicotinamide adenine dinucleotide free acid (NAD) in HEPES buffer, 100 mM adenosine 5'-triphosphate salt hydrate (ATP) in HEPES buffer, NAD dependent Glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* (dilute 1:20 in HEPES buffer), Hexokinase from *Saccharomyces cerevisiae* (dilute 1:30 in HEPES buffer), Phosphoglucose isomerase (PGI) from *Saccharomyces cerevisiae* (dilute 1:10 in HEPES buffer), Invertase from *Saccharomyces cerevisiae* (1 mg/150 µl HEPES buffer). All enzymes were purchased from Merck (Darmstadt, Germany).

Protocol

The initial reaction mix for glucose, fructose and sucrose contained 200 µl HEPES buffer, 10 µl NAD, 10 µl ATP, 10 µl G6PDH and 10 µl water/sample/standard (dilutions of sugar stock). This was first measured at 340 nm for 1 minute to be used as the blank for glucose absorbance. Then 10µl Hexokinase was added and measured at 340 nm for 15 minutes to obtain the glucose absorbance following the reduction of NAD to NADH. This measurement was also used as a blank for fructose absorbance. 10 µl PGI was then added and measured at 340 nm for 15minutes to get the fructose absorbance. This measurement was the blank for sucrose absorbance. 10 µl of Invertase was added and measured at 340 nm for 15 minutes to get sucrose absorbance.

For sugar quantification, a standard curve was prepared using known concentrations of glucose, fructose and sucrose. Each standard solution was measured following the previous procedure and the absorbance values were plotted against the concentration values to generate a linear regression equation. This standard curve was then used to calculate sugar contents of *A. thaliana* samples.

2.4 Statistical Analysis

All data was input to Microsoft Excel spreadsheet, which was used to produce all graphs. Statistical analysis was carried out using R. Flowering time data was assessed using one-way ANOVA to analyse 16 biological replicates (n=16). Eight plants per genotype and treatment were analysed in each of two independent experimental replicates, resulting in a total of 16 plants measured per genotype for each treatment. Mean values were calculated for graphical representation. Control and drought treatments were tested independently. Individual one-way ANOVA tests followed by Tukey HSD post-hoc test were carried out for each measured trait; rosette leaves, cauline leaves and days to flowering. Statistical analysis was not performed on the RT-qPCR data as there was only one experimental replicate. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. A two-way ANOVA followed by Tukey HSD post-hoc test were performed on the starch and soluble sugar data for each individual timepoint, where we compared the effect of genotype, treatment and the interaction between genotype and treatment. Starch and soluble sugar data included 2 experimental replicates with each sample containing the rosettes of two plants.

3.0 Results

3.1 Modulating *lncCDF1/CDF1* levels alters flowering time under drought conditions

Unpublished data identified an early flowering phenotype in *lncCDF1* over-expressing lines suggesting a weak regulatory link between *lncCDF1* and flowering time regulation. Moreover, previous results by the Dr. Henriques research team had shown that *lnccdf1* mutants performed better under drought conditions, therefore to investigate the role of *lncCDF1* in regulating flowering under drought conditions, flowering time was measured in *Arabidopsis thaliana lncCDF1/CDF1* over-expressing and mutant lines. A total of 96 plants (8 plants for each treatment and genotype) per replicate for 2 experimental replicates were grown and assessed under LD conditions at 22°C. Flowering time was recorded as number of days until flowering, and total rosette and cauline leaf number was measured. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test, to compare wildtype (Col-0) with *lncCDF1/CDF1* over-expressing and mutant lines under control and drought conditions analysed independently, where individual ANOVA tests were carried out on each sample; rosette leaves, cauline leaves and days to flowering.

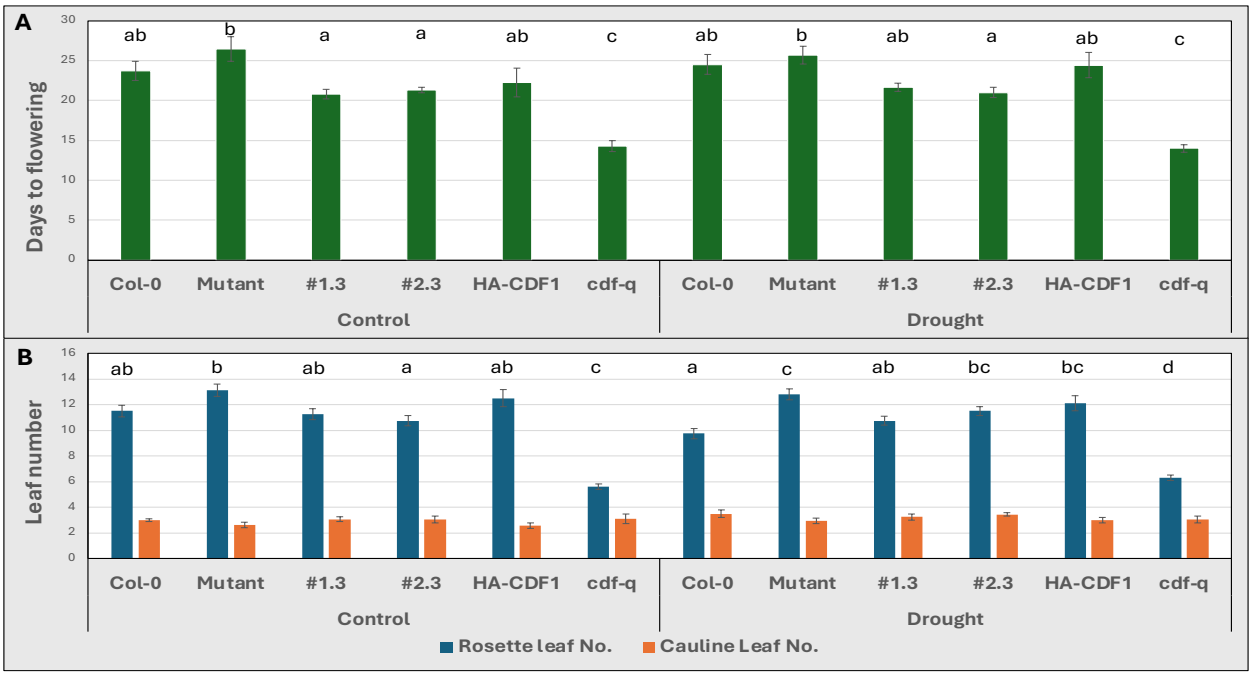


Figure 5: Flowering time assessed as (A) days to flowering and, (B) rosette and cauline leaf number– error bars refer to standard error for all the tested genotypes: wildtype (Col-0), *lnccdf1 salk-mutant #08880-10* (Mutant), *pSUC2::lncCDF1* overexpressor lines (*#1.3* and *#2.3*), *cdf*-quadruple mutant (*cdf-q*) and *HA-CDF1* overexpressor line. All genotypes were tested in two independent experimental replicates (n=16). Significant differences were calculated using one-way ANOVA and Tukey test are denoted via distinct letters ($p<0.05$). Control and drought results were analysed independently, where individual ANOVA tests were carried out on each trait; rosette leaves, cauline leaves and days to flowering.

Analysis of days to flowering under control conditions revealed that there was a significant difference between genotypes ($p<0.005$, Figure 5A). *lncCDF1* overexpressor lines *pSUC2::lncCDF1 #1.3 and #2.3*, and *HA-CDF1* flowered slightly earlier than WT, however this difference was not statistically significant. In contrast, *lncCDF1 Salk-mutant #08880-10* (*lnccdf1* mutant) showed a slightly late flowering phenotype compared to WT, but this was not statistically significant. However, the *lnccdf1* mutant did flower significantly later than *pSUC2::lncCDF1 #1.3 and #2.3* ($p<0.005$, Figure 5A). Our genetic reference, *cdf-q* flowered significantly earlier than WT and all other genotypes, indicated by Tukey test ($p<0.005$, Figure 5A).

Under drought conditions, a one-way ANOVA revealed there was a significant difference in days to flowering between genotypes ($p<0.005$, Figure 5A). Similar to control conditions, *cdf-q* mutant flowers earlier than all genotypes ($p<0.005$, Figure 5A). When exposed to drought both overexpressor lines were shown to flower earlier than WT, but this difference was not statistically significant. Interestingly, under drought conditions only the weaker overexpressor

line *pSUC2::lncCDF1 #2.3* expressed an early flowering phenotype compared with the *lnccdf1* mutant and that was statistically significant ($p<0.05$, Figure 5A).

Analysis of rosette leaf number under control conditions showed a significant difference in rosette leaf number between genotypes ($p<0.005$, Figure 5B). As expected, *cdf-q* produced significantly less rosette leaves than all other genotypes tested ($p<0.005$, Figure 5B), as previously described (Fornara *et al.,* 2009). No other significant differences were noted in comparison to WT. The *lnCDF1 mutant* produced more leaves than both *lncCDF1* overexpressor lines, but only had a statistically significant difference with *pSUC2::lncCDF1 #2.3* ($p<0.005$, Figure 5B).

Under drought conditions, there was a significant difference in rosette leaf number between genotypes ($p<0.005$, Figure 5B). Similar to control conditions, *cdf-q* produced significantly less rosette leaves compared to all other genotypes ($p<0.005$, Figure 5B). *lnccdf1* mutant, *HA-CDF1* and overexpressor line *pSUC2::lncCDF1 #2.3* produced more rosette leaves than WT, which was found to be statistically significant ($p<0.05$, Figure 5B). Interestingly, when exposed to drought, the *lnccdf1* mutant produced more rosettes leaves that both *lncCDF1* over-expressing lines, but only has a significant difference with the strong overexpressor *pSUC2::lncCDF1 #1.3* ($p<0.05$, Figure 5B).

Analysis of cauline leaf number under control conditions using one-way ANOVA revealed no significant differences in cauline leaf number between genotypes (Figure 5B). Using the same test to analyse cauline leaf number under drought conditions no significant differences were found in cauline leaf number between genotypes (Figure 5B).

Taken together, these results provide preliminary evidence that *lncCDF1* may be involved in the regulation of photoperiodic flowering. Although not statistically significant, both overexpressor lines exhibited a trend towards earlier flowering than WT under control and drought conditions. In contrast, the *lnccdf1* mutant tended to take more days to flower than WT under both conditions, consistent with a potential role for lncCDF1 in flowering regulation. Furthermore, in control conditions there is no significant difference in rosette leaf number in the WT compared with *lnccdf1* mutant, *HA-CDF1* and over-expressing lines. However, under drought these genotypes produced significantly more rosette leaves than the wildtype, indicating a delayed transition to flowering or an acceleration of leaf production when exposed to drought. While these trends across genotypes suggests that *lncCDF1* may contribute to

drought-responsive regulation of *CDF1*-dependent flowering, further experiments are required to determine whether this relationship is direct and to establish the underlying mechanisms.

3.2 Drought-responsive expression profiles of *lncCDF1* and *CDF1*

Previous unpublished studies have characterised the expression of *lncCDF1* in both wildtype (Col-0) and *lnccdf1 Salk-mutant #08880-10* (*lnccdf1* mutant) grown under LD conditions *in vitro* at early developmental stages, but this study lacked the characterisation of *lncCDF1* transcript levels under drought stress in older seedlings. The typical expression of lncCDF1 is low at ZT0, then gradually increases during the light phase to a peak between ZT6-ZT9, then declines and remains low for the rest of the day. Real-Time Quantitative PCR (RT-qPCR) was first performed to assess *lncCDF1* transcript levels in wildtype and *lncCDF1 Salk-mutant #08880-10* plants grown in soil under control and drought conditions, across 3 timepoints (ZT1.5, ZT6 and ZT9). To verify the existing gene expression patterns we tested one biological replicate which included two independent plants (Figure 6A). We then broadened the study to test *lncCDF1* transcript levels in both overexpressor lines *pSUC2::lncCDF1 #1.3 and #2.3*, and *HA-CDF1*, when compared with the wildtype (Figure 6B and C).

Similar to *lncCDF1*, previous studies characterised the expression of *CDF1* in wildtype and *lnccdf1* mutant seedlings grown under LD *in vitro.* Therefore, in this study *CDF1* expression levels were also determined in the different *Arabidopsis* genotypes that were exposed to drought stress and grown in soil to complement the previous *in vitro* methods. *CDF1* shows an opposing expression pattern to *lncCDF1* as it starts high at ZT0, then rapidly decreases to reach a trough around ZT9-12. Expression then begins to rise again during the dark phase to prepare for the following day. We followed the same approach as for *lncCDF1* by first characterising *CDF1* expression levels of wildtype and the *lnccdf1* mutant using qPCR (Figure 6D). Following the successful amplification of *CDF1*, we continued to characterise *CDF1* expression patterns in all the *Arabidopsis* genotypes previously tested, including two *lncCDF1* overexpressors *pSUC2::lncCDF1 #1.3 and #2.3*, and *HA-CDF1* grown under control and drought conditions (Figure 6E and F).

3.2.1 *lncCDF1* expression levels

Wildtype (WT) control plants exhibited low *lncCDF1* transcript accumulation at ZT1.5. However, when exposed to drought WT transcript levels almost doubled at this timepoint (Figure 6A, B and C). In contrast to this, *lnccdf1* mutant transcripts peaked under control conditions at ZT1.5, where they were over five times the relative expression of WT control (Figure 6A). Furthermore, when experiencing drought, the *lnccdf1* mutant no longer accumulated high levels of *lncCDF1* transcripts at ZT1.5, with expression levels lower than WT control levels. At ZT6, *lncCDF1* expression increased in WT control, similar to previous results from *in vitro* plants. Under drought conditions WT *lncCDF1* expression levels decreased at ZT6 but remained higher than WT control (Figure 6A). Similarly, in the *lnccdf1* mutant control samples, *lncCDF1* expression declined at ZT6, but was still elevated when compared to WT. Interestingly, the drought exposed *lnccdf1* mutant expression remained low and decreased by nearly 50% at the same timepoint. As expected, at ZT9 *lncCDF1* expression decreased in WT control and showed a greater reduction in the WT drought sample. A similar response was observed in the *lnccdf1* mutant which showed lower *lncCDF1* expression levels at ZT9 than the WT sample. At the same timepoint, we observed extremely low expression levels in the *lnccdf1* mutant under drought conditions, which were also lower than WT control (Figure 6A).

I then performed a parallel experiment where we tested *lncCDF1* expression levels again and observed the WT control to follow a similar *lncCDF1* expression pattern as in the first replicate (Figure 6A, B and C). Under drought conditions, we found slight variances in WT at ZT6, where *lncCDF1* expression was lower in WT drought samples in this second replicate. Unsurprisingly, both overexpressor lines had higher *lncCDF1* expression levels throughout all timepoints compared with WT control (Figure 6B and C). As expected, the stronger overexpressor *pSUC2::lncCDF1 #1.3* has the highest *lncCDF1* expression levels, peaking at ZT1.5 under control conditions. This decreased under drought conditions at the same timepoint, but still remains high when compared with WT. The second overexpressor line *pSUC2::lncCDF1 #2.3* has lower *lncCDF1* expression levels under control conditions compared to *pSUC2::lncCDF1 #1.3* at ZT1.5. However, under drought stress there was a significant increase of *lncCDF1* expression in *pSUC2::lncCDF1 #2.3*, showing transcript levels similar to the stronger overexpressor (*pSUC2::lncCDF1 #1.3*) under the same conditions. When the *HA-CDF1* overexpressor line was analysed under control conditions it showed higher *lncCDF1* expression (2x increase) when compared with WT. This expression

induction further increased in *HA-CDF1* seedlings grown under drought conditions, with transcript levels similar to that of *pSUC2::lncCDF1 #2.3* (6-fold relative expression) control at ZT1.5 (Figure 6B and C).

We observed a strong decrease of *lncCDF1* expression in the *pSUC2::lncCDF1 #1.3* overexpressor at ZT6 (approx. 4.5x) under control conditions, although transcript levels in this line remained considerably higher than WT (Figure 6C). Under drought conditions the *pSUC2::lncCDF1 #1.3* line accumulated lower *lncCDF1* levels when compared with ZT1.5. No big change was observed in *pSUC2::lncCDF1 #2.3* under control conditions, but drought plants exhibited a 3x decrease of expression. Furthermore, in *HA-CDF1* seedlings expression levels increase at ZT6, staying consistently higher than WT (approx. 5x). In contrast, in the drought exposed *HA-CDF1* seedlings, *lncCDF1* transcript levels substantially decrease when compared with control conditions and were also lower than WT control at the same time point (Figure 6C).

At ZT9, we observed that *lncCDF1* expression remained the same (approx. 1-fold relative expression) in WT seedlings under both control and drought conditions. However, in both overexpressor lines (*pSUC2::lncCDF1 #1.3* and *#2.3*) there was an increase in *lncCDF1* expression under control conditions (Figure 6B and C). However, in the strong overexpressor (*pSUC2::lncCDF1 #1.3)*, *lncCDF1* transcript levels increased when compared with ZT6. However, when analysing ZT9 conditions, we observed 2x higher *lncCDF1* levels in control seedlings when compared with drought conditions. In the weaker line *pSUC2::lncCDF1 #2.3* we observed a similar increase at this timepoint. In this line however, *lncCDF1* expression was slightly higher under drought conditions when compared with the control. Moreover, *HA-CDF1* control plants showed a decreased in *lncCDF1* transcript levels, but remain upregulated compared with WT at ZT9. When treated with drought stress *HA-CDF1* plants greatly increased *lncCDF1* expression, although these levels were remained lower than the *lncCDF1* overexpressors (Figure 6C).

Together, these results demonstrate how *lncCDF1* transcription is responsive to drought stress. The contrasting expression patterns of *lncCDF1* observed across genotypes, in particular the drought induced activation in *pSUC2::lncCDF1 #2.3* and suppression in *lnccdf1* mutant, indicate that *lncCDF1* regulation may be influenced by environmental conditions. While these observations are consistent with a potential role for *lncCDF1* in drought responses, further

investigation is required to determine whether the observed changes in expression contribute directly to drought tolerance or reflect downstream responses to stress.

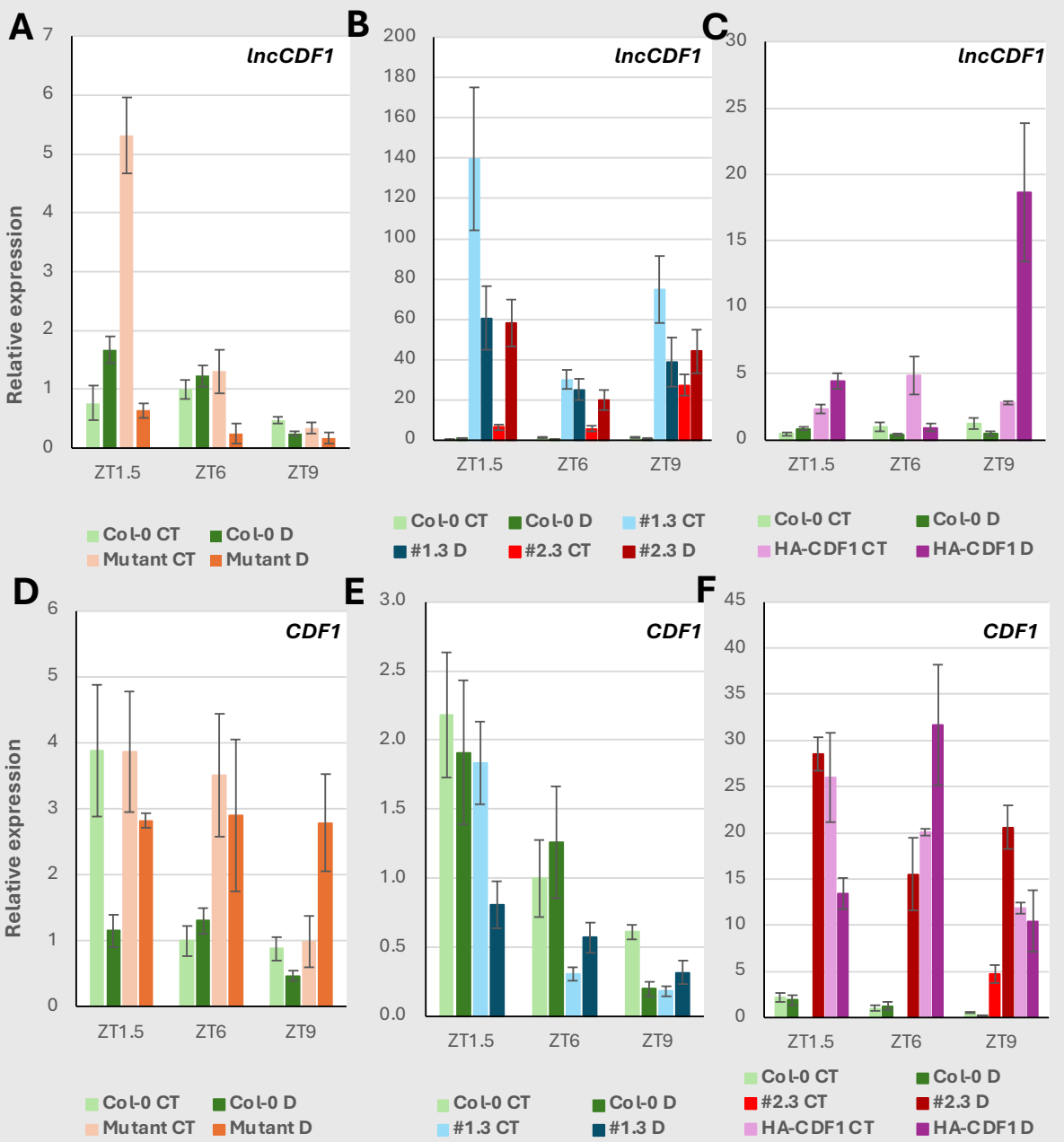


Figure 6: Relative expression levels of (A) *lncCDF1* in wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0), and *pSUC2::lncCDF1* overexpressor line (*#1.3*) grown under CT and D conditions; (C) wildtype (Col-0), *pSUC2::lncCDF1* overexpressor line (*#2.3*) and *HA-CDF1* overexpressor line grown under CT and D conditions. (D) *CDF1* relative expression in wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD CT and D conditions; (E) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor lines (*#1.3* and *#2.3*) grown under CT and D conditions; (F) wildtype (Col-0) and *HA-CDF1* overexpressor line grown under CT and D conditions. Wildtype (Col-0) *lncCDF1* and *CDF1* expression values used in the middle and right panels are the same for both conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels and error bars refer to standard deviation of 3 technical replicates from 1 experimental replicate.

3.2.2 *CDF1* expression levels

In parallel to the *lncCDF1* expression analysis, we also investigated how *CDF1* transcripts accumulate in the different genotypes tested. At ZT1.5 we observed similar *CDF1* expression levels in both WT and *lnccdf1* mutant under control conditions (Figure 6D). When exposed to drought stress *CDF1* expression decreases 4x in WT seedlings, but only slightly in the mutant. At ZT6 we observed a different pattern of *CDF1* accumulation in both genotypes under control conditions, whereas in the WT there was a 4x decrease, the *lnccdf1* mutant maintained a similar expression level to Col-0 at ZT1.5. Under drought conditions, *CDF1* expression was similar to the control conditions at this time point, with either a slight increase or decrease in the WT and *lnccdf1* mutant, respectively. (Figure 6D).

At ZT9, WT plants grown under control conditions accumulated *CDF1* transcript levels which were 4x lower than at ZT1.5, but similar to ZT6. In WT drought treated plants, *CDF1* levels were 3x lower than those of control plants (Figure 6D). In the *lnccdf1* mutant seedlings grown under control conditions, *CDF1* expression was similar to WT at ZT9, but 4x lower than at ZT1.5. Notably, in the drought treated *lnccdf1* mutant , *CDF1* transcript accumulation remained consistently higher, similar to ZT1.5 and ZT6, suggesting that these seedlings maintain high *CDF1* levels when exposed to this abiotic stress (Figure 6D).

When analysing the *lncCDF1* overexpressor lines, we observed that WT control plants exhibited a *CDF1* expression profile consistent with the pattern described earlier, showing a gradual decline of expression across the three timepoints (Figure 6D, E and F). We observed a slight difference in wildtype drought-treated plants, where *CDF1* expression is now slightly higher at ZT1.5 than at ZT6 as observed in the first experiment (Figure 6D and E). The overexpressor line *pSUC2::lncCDF1 #1.3* grown under control conditions had similar *CDF1* expression levels to that of WT drought plants at ZT1.5. This expression decreases in *pSUC2::lncCDF1 #1.3* plants exposed to drought at the same timepoint. Expression of *CDF1* could not be quantified in the weaker overexpressor line *pSUC2::lncCDF1 #2.3* grown under control conditions at ZT1.5, with high Ct values suggesting very low transcript accumulation. However, this was not the case in *pSUC2::lncCDF1 #2.3* drought plants, where we observed peak expression of *CDF1* at ZT1.5 with levels significantly greater than wildtype under similar conditions (over 9x). These remained high at ZT6 and ZT9, being 15x and 5x of WT plants, respectively. Similarly, in *HA-CDF1* seedlings grown under control conditions at ZT1.5 there were high expression levels of *CDF1* (approx. 9x) when compared with WT at the same time

point. However, under drought conditions *CDF1* expression levels decreased but still higher than the WT grown under similar conditions (approx. 5x) (Figure 6E and F).

At ZT6 we observed a decrease of *CDF1* expression in both *pSUC2::lncCDF1 #1.3* control and drought plants, though a greater decline of expression was observed in control plants (over 6x). *CDF1* expression at ZT6 in *pSUC2::lncCDF1 #2.3* could not be quantified as similar to ZT1.5 Ct values were high, possible due to low transcript accumulation (Figure 6E). However, drought treated *pSUC2::lncCDF1 #2.3* maintained higher expression levels compared with WT, but decreased from ZT1.5 to ZT6. We also observed a similar trend in *HA-CDF1* control plants, as *CDF1* expression remains high but decreased from ZT1.5. Contrastingly, *CDF1* transcript levels almost double in *HA-CDF1* drought plants at ZT6 (Figure 6F).

At ZT9 there was little change in *CDF1* expression levels in *pSUC2::lncCDF1#1.3* under control and drought conditions (Figure 6E). However, at ZT9 there were higher levels of *CDF1* expression in *pSUC2::lncCDF1 #2.3* (5x) compared with WT under control conditions. When *pSUC2::lncCDF1 #2.*3 seedlings were exposed to drought, *CDF1* expression increased to more than 20x when compared with WT seedlings in similar conditions. The *HA-CDF1* line showed similar *CDF1* accumulation under both control and drought conditions, but its transcript levels were still considerably higher than WT (approx. 20x). This decrease is in greater effect in drought treated *HA-CDF1*, as expression levels drop below control plants at the same timepoint (Figure 6F).

Collectively, these observations indicate that *CDF1* expression is affected by drought stress and *lncCDF1* mutations. In agreement with *CDF1* circadian oscillation, wildtype plants accumulated less *CDF1* transcript as the day progressed, whereas drought conditions altered the expression profile. Contrastingly, the *lnccdf1* mutant accumulated comparable *CDF1* transcript levels at ZT1.5 and 6 both in control and drought conditions. At ZT9 we observed higher *CDF1* accumulation only in drought exposed mutants. Overexpressor lines showed opposite expression profiles, with *pSUC2::lncCDF1 #1.3* showing low *CDF1* expression and *pSUC2::lncCDF1 #2.3* exhibiting strong drought responsive peaks. As expected, *HA-CDF1* seedlings accumulated elevated *CDF1* levels in control plants, but had variable drought responses throughout the day. Taken together, these expression patterns suggest that misexpression or overexpression of *lncCDF1* can lead to shifts in *CDF1* transcript dynamics under control and drought conditions.

3.3 qPCR-based expression profiling of drought responsive genes in *Arabidopsis*

Previous work by Dr. Henriques and colleagues identified, using RNA-Seq, multiple genes that experienced the greatest change in expression under drought conditions in *A. thaliana*, these included; *QUA QUINE STARCH (QQS)*, *SUCROSE-PROTON SYMPORTER 7 (SUC7)*, *OSMOTIN 34 (OSM34)*, *EXTENSIN 1 (EXT1)*, and *MOTHER OF FT AND TFL (MFT)*. *QQS* showed a down regulation in expression under drought conditions, whereas the remaining four genes displayed an upregulation in expression of varying severity. However, these findings only highlight the expression at a single timepoint (ZT6). To validate the identification of these *lncCDF1*-regulated genes, we used qPCR to characterise their expression in wildtype and *lnccdf1 salk-mutant #08880-10* (*lnccdf1* mutant) at the same 3 timepoints as previously described (ZT1.5, ZT6 and ZT9). Following successful amplification of all genes we expanded this study to profile the expression of these candidate genes in two *lncCDF1* overexpressor lines *pSUC2::lncCDF1 #1.3* and *#2.3*, as well as in *CDF1* overexpressor plants *HA-CDF1*.

3.3.1 *QQS* expression levels

The orphan gene *QQS* is involved in modulating carbon and nitrogen allocation, where overexpression of *QQS* decreases starch content in the leaves of *A. thaliana* (Li *et al.,* 2015). At early morning (ZT1.5) *QQS* expression levels are highest in WT control plants whereas in drought treated plants its expression decreased (Figure 7A). The *lnccdf1* mutant accumulated lower QQS levels (3.5x less) under both control and drought conditions when compared to WT. At ZT6, the expression of *QQS* increased in WT control plants, as opposed to the decline in expression in drought plants (approx. 0.5x less than CT conditions). However, the *lnccdf1* mutant showed an upregulation of *QQS* for both treatments (approx. a 2x increase), but its expression levels remained lower than WT (Figure 7A).

In contrast to ZT6, at ZT9 WT control *QQS* expression decreased but it increased in drought plants, maintaining similar expression to control. Furthermore, the *lnccdf1* mutant control sample showed very low expression levels at ZT9 whereas seedlings grown under drought conditions, although with a slight lower expression than at ZT6, still maintained *QQS* transcript levels similar to the WT (Figure 7A).

The second experiment confirmed a similar *QQS* expression pattern in WT control plants. In drought conditions there was a slight difference in expression as we observed higher expression levels at ZT6 as opposed to ZT9 compared with the first experiment. At ZT1.5 both over expressors *pSUC2::lncCDF1 #1.3 and #2.3*, and *HA-CDF1* had lower *QQS* transcript levels compared with WT. Similar to wildtype, a down regulation of *QQS* expression was observed in these genotypes when exposed to drought (Figure 7B).

At ZT6, all genotypes maintained lower expression levels of *QQS* compared with WT control. However, although *QQS* transcript levels were reduced (approx. 3x) in WT plants under drought conditions, in the stronger overexpressor *pSUC2::lncCDF1 #1.3 QQS* expression levels were similar for both treatments. Similarly, there were no big changes in transcript accumulation for the second overexpressor *pSUC2::lncCDF1 #2.3* under control and drought conditions, although *QQS* transcript levels were lower than in line *pSUC2::lncCDF1 #1.3* . *HA-CDF1* exhibited a decline in expression in control plants, whereas drought treated plants expression levels are upregulated (Figure 7B).

At ZT9 both *pSUC2::lncCDF1 #1.3 and #2.3* control plants experienced a decline of *QQS* expression, maintaining levels lower than that of WT control. Interestingly, *QQS* is upregulated under drought stress in both over expressing genotypes. There appeared to be no change of expression levels in *HA-CDF1* control plants, but we observe a decline in expression in drought treated plants with *QQS* levels similar to that of the control (Figure 7B).

Overall, analysis of *QQS* expression revealed that WT plants exhibit a diurnal and drought responsive pattern, with strong induction early in the light period and a clear repression under drought conditions. In contrast, the *lnccdf1* mutant maintained low expression, with a modest response to drought suggesting a reduced capacity to regulate *QQS*. The examination of overexpressing lines and *HA-CDF1* plants further supports the notion that misexpression of *lncCDF1* influences *QQS* transcriptional behaviour, as we observed low expression levels with genotype-specific increases in drought treated plants. Together, these findings highlight the effect *lncCDF1* has on *QQS* expression dynamics, reinforcing the broader role of *lncCDF1*-mediated regulation within stress-responsive transcriptional networks.

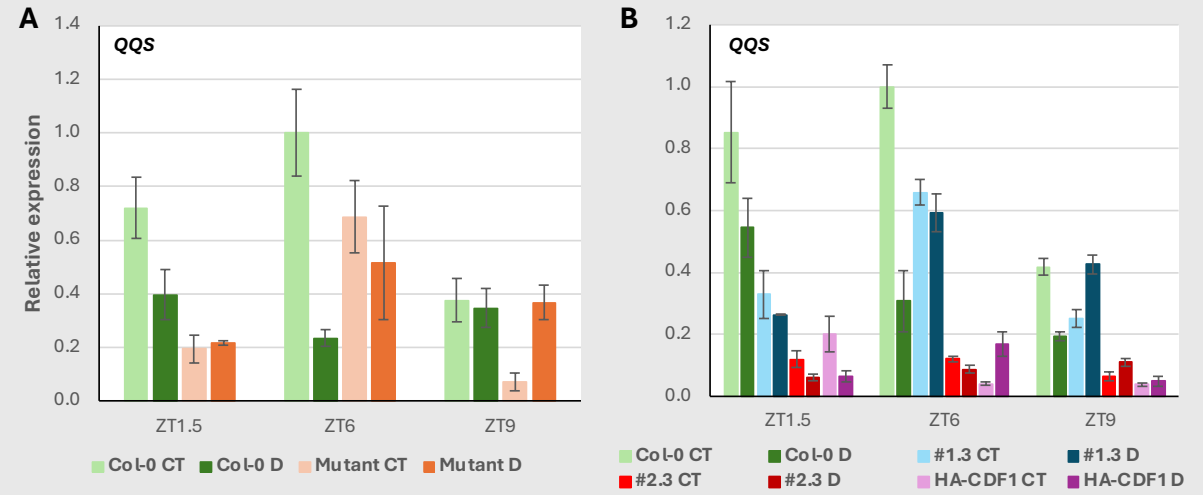


Figure 7: Relative expression levels of *QQS* in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0), *pSUC2::lncCDF1* overexpressor lines (*#1.3 and #2.3*) and *HA-CDF1* overexpressor line under CT and D conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels in three technical replicates, and lines refer to SD values of 3 technical replicates from 1 experimental replicate.

3.3.2 *SUC7* expression levels

Studies suggest that *SUC7* is a sucrose proton symporter, but lacks the ability to mediate the transport of sugars across the plasma membrane (Rottmann *et al.,* 2018). Indicating that SUC7 is a non-functional pseudogene but potentially plays a more important role in sugar signalling. Expression of *SUC7* is low in WT control plants at ZT1.5 but shows an upregulation of expression (approx. 4x) under drought conditions (Figure 7A). Expression levels were higher in *lnccdf1* mutant control plants when compared with wildtype, but there was a similar upregulation of *SUC7* expression under drought conditions.

At ZT6, *SUC7* expression decreases both in WT control and drought plants, but its levels remained upregulated in drought plants. Interestingly, when compared with ZT1.5 we observed an increase of *SUC7* transcripts in the *lnccdf1* mutant control sample, while the drought treated mutant exhibited a decrease in its expression (Figure 8A).

At ZT9, the WT control displayed its peak expression of *SUC7*, but this was still lower (approx. 2x) in comparison to the *lnccdf1* mutant control plants. Drought exposed WT plants also experienced a peak in *SUC7* expression, being double of that of the control. The *lnccdf1* mutant also had peak *SUC7* expression at this time point, showing expression levels similar to WT drought plants. However, under drought conditions we observed an extreme upregulation in

SUC7 expression, which is 6x higher than in mutants grown under control conditions (Figure 8A).

At ZT1.5, the second experiment revealed *SUC7* expression levels in WT control and drought grown plants which were quite similar to the first experiment (Figure 8). When we analysed *lncCDF1* overexpressor lines, we found that control *pSUC2::lncCDF1 #1.3* plants had higher *SUC7* expression levels than WT control samples (approx. 2x). Although, WT plants showed an up-regulation of SUC7 expression under drought conditions, the overexpressor line maintained similar levels of this transcript in control and drought conditions (Figure 8B and C). However, in the second overexpressor line *pSUC2::lncCDF1 #2.*3 the opposite occurred as we observed down regulation of *SUC7* accumulation under drought conditions. *HA-CDF1* plants, on the other hand, accumulated the lowest levels of *SUC7*, which were comparable both in control and drought conditions.

At ZT6, WT samples, both control and drought, showed an overall reduction in *SUC7* expression (approx. 4x and 3.5x, respectively) when comparing with ZT1.5. *pSUC2::lncCDF1 #1.3* plants accumulated comparable amounts of *SUC7* transcripts under both control and drought conditions, but these were still higher than the corresponding WT samples. However, *pSUC2::lncCDF1 #2.3* showed a decrease in *SUC7* expression, and its levels were lower in drought plants . *HA-CDF1* showed an increase in *SUC7* transcript levels, but under drought conditions there was a down regulation of its expression (Figure 7B and C).

At ZT9, *SUC7* expression increased in WT control plants, and it maintained its high levels under drought conditions (Figure 8B and C). *pSUC2::lncCDF1 #1.3* control plants showed a decline in *SUC7* expression (approx. 6x), whereas in drought treated plants its expression is upregulated (approx. 2.5x) . However, both *pSUC2::lncCDF1 #2.3* and *HA-CDF1* plants, display very low *SUC7* expression levels, with little change between control; and drought.

Taken together, these results demonstrate how *SUC7* expression is strongly influenced by drought stress and *lncCDF1* accumulation. WT plants consistently showed drought-induced upregulation of *SUC7*, whereas the *lnccdf1* mutant maintained elevated baseline expression but displayed a varied transcriptional response to drought, especially at ZT9. Over-expressing lines showed more variability among each other, highlighting the differences in overexpression strength. Moreover, *HA-CDF1* showed generally low levels of *SUC7* with limited responsiveness to drought. Overall, the data indicates that all lines exhibit unique temporal

dynamics in *SUC7* accumulation with varied responses to drought, reinforcing the role of *lncCDF1* in mediating drought stress responses.

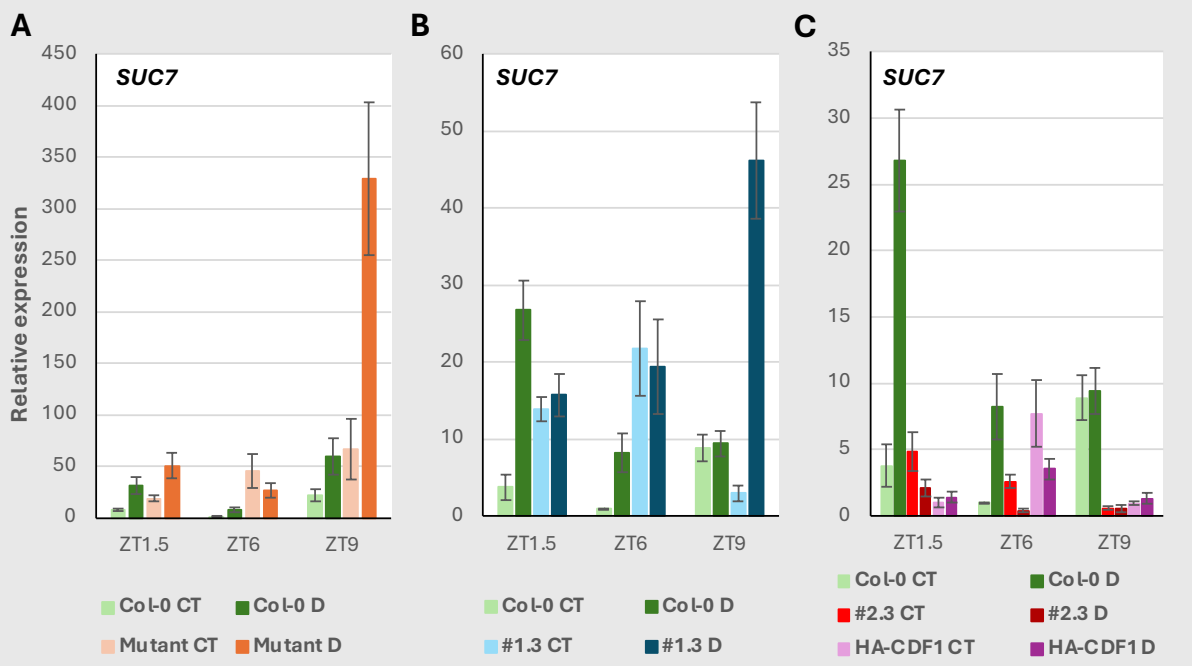


Figure 8: Relative expression levels of *SUC7* in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor line (*#1.3*) grown under CT and D conditions; (C) wildtype (Col-0), *pSUC2::lncCDF1* overexpressor line (*#2.3*) and *HA-CDF1* overexpressor line under CT and D conditions. Wildtype (Col-0) *SUC7* expression values used in the (B) and (C) panels are the same for both conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels in three technical replicates, and lines refer to SD values of 3 technical replicates from 1 experimental replicate.

3.3.3 *OSM34* expression levels

OSM34 is described as a positive regulator of ABA responses that is under post-translational control (Park *et al.,* 2021). WT control exhibited very low *OSM34* expression levels throughout the time course ZT1.5-9, with little change between the timepoints. However, *OSM34* expression was upregulated in drought treated plants, and levels also increased throughout the day (Figure 9A). At ZT1.5, the *lnccdf1* mutant accumulated more *OSM34* transcripts (approx. 14x) than WT plants under control conditions, whereas drought exposed plants experienced a clear upregulation of expression (approx. 9x). The *lnccdf1* mutant maintained *OSM34* expression levels higher than WT at ZT6, both in control and drought conditions. Interestingly, drought treated plants experienced a decline in expression compared with ZT1.5 suggesting a downregulation of *OSM34*. Furthermore, the *lnccdf1* mutant continued to show an increase in

OSM34 expression at ZT9, with a slight upregulation of when the mutants are exposed to drought (Figure 9A).

When this experiment was repeated, we continued to observe the same expression pattern of *OSM34* in WT plants, with a consistent upregulation of expression in drought exposed plants from ZT1.5 until ZT9 (Figure 9A and B).

At ZT1.5, *OSM34* expression is elevated in *pSUC2::lncCDF1 #1.3* control plants compared with wildtype, whereas *pSUC2::lncCDF1 #2.3* exhibited expression levels similar to WT. However, both overexpressor lines showed upregulated *OSM34* expression under drought conditions. Similarly to *pSUC2::lncCDF1 #2.3, HA-CDF1* control plants displayed expression levels resembling the WT, but there was some upregulation of *OSM34* expression in drought treated plants (Figure 9B).

At ZT6, *OSM34* expression levels remained elevated in *pSUC2::lncCDF1 #1.3* both under control and drought conditions (Figure 9B). We observed no significant changes in its expression in *pSUC2::lncCDF1 #2.3*, although drought treated plants showed a slight down-regulation of this transcript .Moreover, *HA-CDF1* had an increase in *OSM34* expression, especially in drought treated plants, following the same trend as at early morning ZT1.5.

OSM34 greatly decreased in *pSUC2::lncCDF1 #1.3* control plants at ZT9, whereas drought pants displayed peak expression. We observed little change in *OSM34* transcript levels in *pSUC2::lncCDF1 #2.3* control and drought plants, as it follows the same pattern as seen at ZT6. *HA-CDF1* plants maintained an upregulation of *OSM34* expression under drought conditions, but did exhibit an overall decline of expression when compared with ZT6 (Figure 9B).

Expression analysis of *OSM34* revealed a drought-responsive pattern across all genotypes. WT expression of *OSM34* is consistently upregulated under drought conditions throughout the entire time course. The *lnccdf1* mutant displayed a more dynamic response to drought, although maintaining elevated expression levels under control conditions. Over-expressing lines showed genotype-specific differences, whereas *HA-CDF1* expression patterns resembled the wildtype. Collectively, these results help understand the role *lncCDF1* and *CDF1* in drought induced responses, and highlight distinct regulatory behaviours among different genotypes.

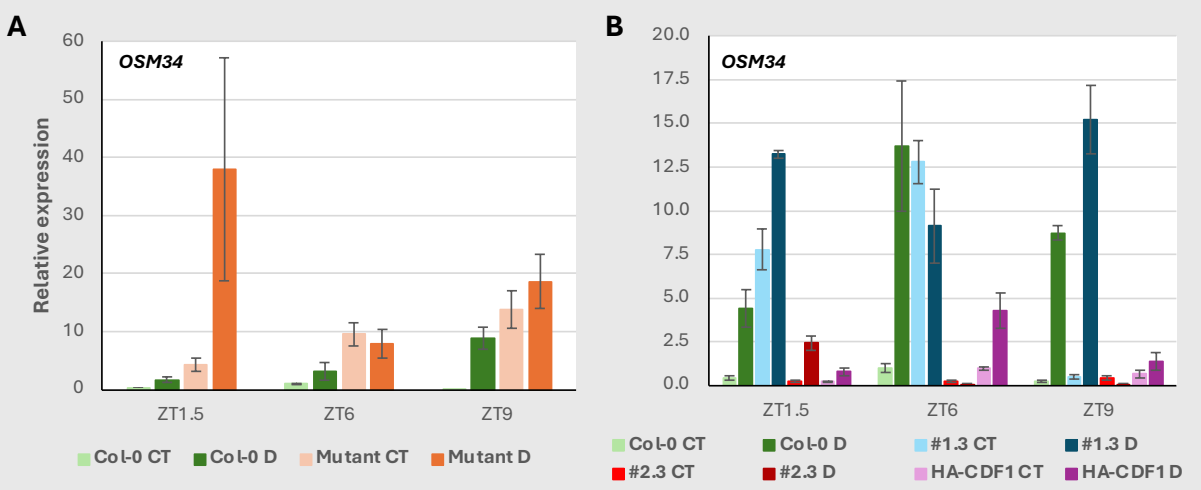


Figure 9: Relative expression levels of *OSM34* in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0), *pSUC2::lncCDF1* overexpressor lines (*#1.3 and #2.3*) and *HA-CDF1* overexpressor line under CT and D conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels in three technical replicates, and lines refer to SD values of 3 technical replicates from 1 experimental replicate.

3.3.4 *MFT* expression levels

MFT is part of the phosphatidylethanolamine-binding protein (PEBP) family, functioning as a regulator of flowering time and is a homolog of *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) (Xi *et al.,* 2010). WT control displayed the highest expression levels of *MFT* at ZT1.5, with a clear down regulation in drought plants (Figure 10A). In contrast, the *lnccdf1* mutant has low *MFT* expression levels, with a visible upregulation under drought conditions. At ZT6, we observed a decrease of *MFT* expression in WT control, whereas drought plants increased transcript levels, when compared with ZT1.5 . *MFT* expression levels did not differ from ZT1.5 in *lnccdf1* mutant control plants but there was a stronger up-regulation in drought treated plants. We observed peak expression of *MFT* in WT control plants at ZT9, whereas drought exposed plants maintained similar transcript levels to ZT6. Similarly, *lnccdf1* mutant control plants also exhibit peak expression at this time point, although it was lower in comparison to WT. However, *MFT* transcripts could not be detected under drought conditions possibly due to high Ct values or technical error (Figure 10A).

The second experiment revealed an overall higher expression of *MFT* (compare scales of all panels in Figure 10). Although its expression profiles remained consistent in WT plants, there was a slight change at ZT6, where *MFT* expression levels were now higher in drought plants (Figure 10).

At ZT1.5 *pSUC2::lncCDF1 #1.3* control plants appeared to have higher expression levels than WT, whereas the weaker overexpressor *pSUC2::lncCDF1 #2.3* showed a much greater increase in *MFT* expression (Figure 10B and C). However, both overexpressors appeared to repress *MFT* expression under drought conditions. In contrast, *HA-CDF1* expression levels were lower in the control but increase in drought treated plants.

At ZT6 expression levels of *MFT* in *pSUC2::lncCDF1 #1.3* control did not differ from early morning, but there was an upregulation of its expression in drought plants, as oppose to the repression observed at ZT1.5 (Figure 10B and C). We observed a similar trend in *pSUC2::lncCDF1 #2.3* seedlings with a decrease in expression in control plants, but an upregulation in drought plants. *HA-CDF1* plants exhibited elevated expression levels compared with WT, both in control and drought conditions, maintaining the same trend as the previous timepoint.

At ZT9 both overexpressor lines grown under control conditions accumulated *MFT* transcript levels that were similar to ZT6 (Figure 10B and C). However, under drought conditions, *pSUC2::lncCDF1 #1.3* plants showed similar expression levels to its control, whereas *pSUC2::lncCDF1 #2.3* showed a peak in *MFT* expression over 10-fold to that of its control. In *HA-CDF1* lines we observed a similar *MFT* expression pattern to ZT6 , although under drought conditions there was a slight increase.

Examination of *MFT* expression revealed distinct genotype-specific expression profiles. WT plants exhibit drought induced repression of *MFT*. The *lnccdf1* mutant exhibited consistently low levels of expression under control conditions, with a clear drought-induced upregulation at early timepoints. Both overexpressors show a downregulation regulation of *MFT* expression in drought plants at ZT1.5, before shifting to an upregulation at the later timepoints. In contrast, *HA-CDF1* maintained a consistently increasing upregulation of *MFT* expression under drought conditions. Overall, these results further support the role of *lncCDF1/CDF1* in modulating drought stress responses in *A. thaliana*.

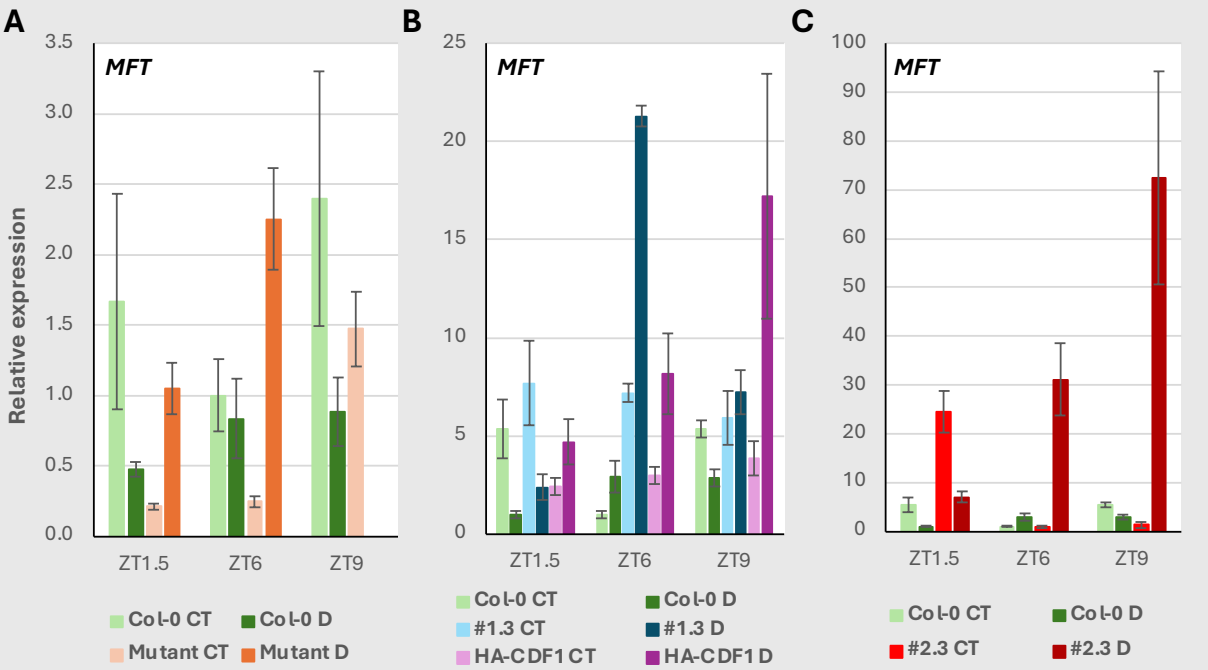


Figure 10: Relative expression levels of *MFT* in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0), *pSUC2::lncCDF1* overexpressor lines(*#1.3*) and *HA-CDF1* overexpressor line grown under CT and D conditions; (C) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor line (*#2.3*) grown under CT and D conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels in three technical replicates, and lines refer to SD values of 3 technical replicates from 1 experimental replicate.

3.3.5 *EXT1* expression levels

EXT1 is generally related to pathogen responses as it is a cell-wall hydroxyproline-rich glycoprotein which enforces cell wall strength (Wei and Shirsat, 2006). In WT control plants *EXT1* expression stayed relatively low throughout all timepoints (Figure 11). However, when grown under drought conditions there was an upregulation of expression, which was stronger (approx. 8x) at ZT9. At ZT1.5 *EXT1* expression was slightly elevated in the *lnccdf1* mutant compared to wildtype under control conditions, but its expression was upregulated under drought. At ZT6, however, both control and drought treated plants accumulated similar levels of *EXT1*, which reflected an induction under control conditions but no change under drought. AT ZT9, *lnccdf1* mutant plants accumulated peak levels of *EXT1* both under control and drought conditions (figure 11A).

The second experiment also confirmed the expression pattern of *EXT1* in WT control and drought plants, as trends remained consistent in both (Figure 11A and B).

At ZT1.5, *pSUC2::lncCDF1 #1.3* control plants exhibited strong *EXT1* expression levels compared to WT (Figure 11B). However, under drought conditions there was a down

regulation of *EXT1* expression by over half, which was comparable to WT drought samples. Both lines *pSUC2::lncCDF1 #2.3* and *HA-CDF1* showed consistently low expression levels of *EXT1* when compared with both WT control and drought conditions (Figure 11C).

At ZT6, *pSUC2::lncCDF1 #1.3* showed peak *EXT1* expression in control plants, but it accumulated at 14x lower levels in drought treated plants (Figure 11B and C). *EXT1* expression was low in *pSUC2::lncCDF1 #2.3* plants grown under either control or drought conditions. We observed no major change in expression in *HA-CDF1* control plants when comparing to ZT1.5, but in drought plants its expression increased.

At ZT9, *EXT1* transcript levels decreased 15x in *pSUC2::lncCDF1 #1.3* control plants, but it accumulated at similar levels to ZT6 in drought treated plants. In the line *pSUC2::lncCDF1 #2.3* expression levels of *EXT1* remained low, consistent to those of ZT6. We observed a similar result in *HA-CDF1* plants, with drought treated plants exhibiting a stronger reduction in *EXT1* transcript levels (Figure 11B and C).

These results show that *EXT1* is minimally expressed in WT plants, but is upregulated under drought stress, especially at ZT9. The *lnccdf1* mutant displayed elevated basal expression, although at later timepoints *EXT1* expression is down regulated in drought treated plants. *LnCDF1* overexpressor lines exhibited divergent expression patterns, where *pSUC2::lncCDF1 #1.3* showed increased *EXT1* expression under control conditions, but *pSUC2::lncCDF1 #2.3* maintained low expression levels of this gene. Moreover, drought treated *pSUC2::lncCDF1 #1.3* plants exhibited a clear repression of *EXT1* transcription in the morning, whereas *pSUC2::lncCDF1 #2.3* showed reduced responsiveness to drought. Similar to WT, *HA-CDF1* maintained low *EXT1* expression levels, with only a slight drought induced upregulation at ZT6. Collectively, this data continues to reinforce the influence *lncCDF1* levels have on drought stress regulatory networks.

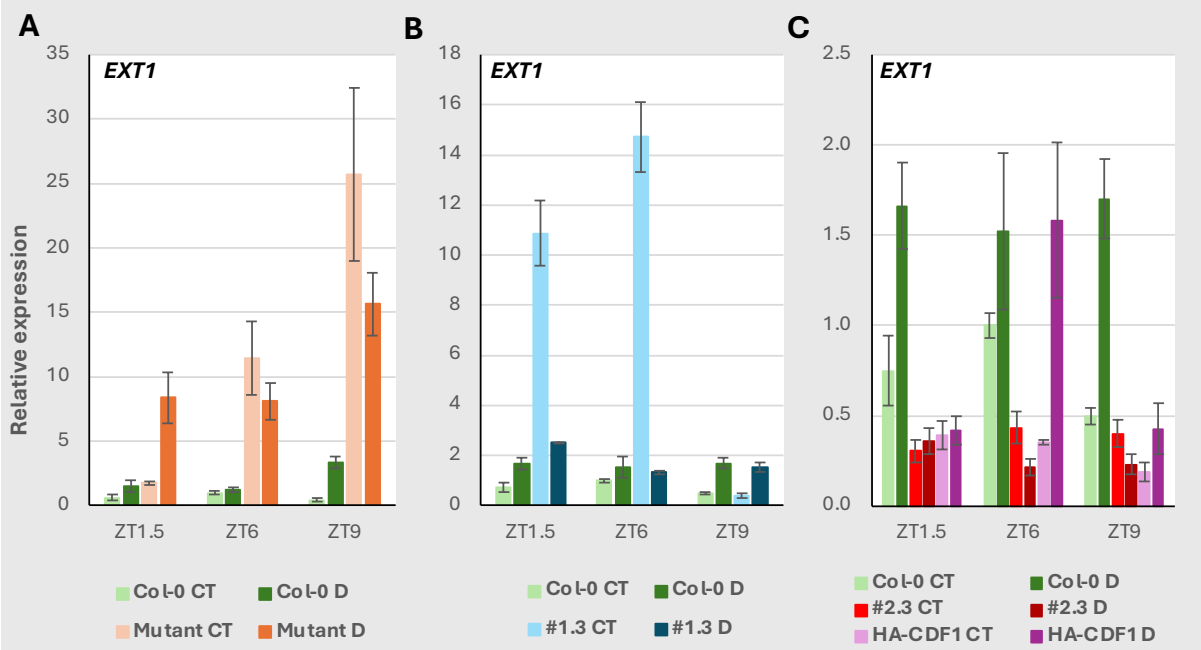


Figure 11: Relative expression levels of *EXT1* in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) grown under LD control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor line (*#1.3*) under CT and D conditions; (C) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor line (*#2.3*) and *HA-CDF1* overexpressor line under CT and D conditions. Wildtype (Col-0) *EXT1* expression values used in the (B) and (C) are the same for both conditions. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method where all expression values were compared to the sample Col-0 CT ZT6 which was assigned a value of 1. Bars represent relative expression levels in three technical replicates, and lines refer to SD values of 3 technical replicates from 1 experimental replicate.

3.4 Modulation of starch and soluble sugars under drought conditions

After the characterisation of drought-responsive gene expression, we sought to investigate whether these transcriptional changes of genes involved in sugar transport and metabolism would translate into altered starch and soluble sugar levels under both control and drought conditions. We determined the levels of starch and soluble sugars in the 5 studied genotypes; Col-0*, lnccdf1 Salk-mutant #08880-10* (*lnccdf1* mutant)*, pSUC2::lncCDF1 #1.3* and *#2.3*, and *HA-CDF1*. Each line was grown in control and drought conditions and collected at 4 timepoints ZT1.5, ZT6, ZT9 and ZT16. We combined the rosettes of two plants per sample for a total of 2 experimental replicates. For the purpose of this study, we analysed each line separately, comparing every genotype to wildtype to clearly evaluate genotype specific differences. First analysing wildtype with the *lnccdf1* mutant, then wildtype with both overexpressors and finally wildtype with *HA-CDF1.*

3.4.1 Starch

Analysis of starch levels revealed moderate starch accumulation in WT peaking at ZT16, consistent with typical diurnal starch metabolism (Figure 12A). We observed little differences in starch accumulation between WT plants grown under control and drought conditions across all timepoints. The *lnccdf1* mutant control plants generally showed lower starch levels than WT, in particular at later timepoints such as ZT9 and ZT16. Starch accumulation was higher under drought conditions in the *lnccdf1* mutant, but this different was not statistically significant.

Over-expressing lines grown under control conditions showed divergent starch patterns, as *pSUC2::lncCDF1#1.3* showed low levels at early morning, but these increased and remained consistent throughout the day, whereas *pSUC2::lncCDF1 #2.3* plants accumulated the highest starch values peaking at ZT6 (Figure 12B). In drought treated plants, *pSUC2::lncCDF1 #1.3* accumulated different starch levels, which were higher than the control at ZT1.5 and ZT16 but lower at ZT6 and ZT9. *pSUC2::lncCDF1 #2.3* generally showed lower levels of starch under drought conditions, in particular ZT1.5 and ZT6 (Figure 12B).

The *HA-CDF1* line grown under control conditions displayed a unique starch profile, with clear peaks at ZT1.5 and ZT9 which were greater than the WT (Figure 12C). However, starch levels were considerably lower than WT at ZT6 and ZT16. Drought appeared to only affect starch accumulation at the early morning timepoints, where we observed a decrease of starch at ZT1.5, but and increase at ZT6 (Figure 12C).

Analysis was carried out using two-way ANOVA, where we tested the effect of genotype, treatment and the interaction between genotype and treatment. The results revealed that treatment and the interaction between genotype and treatment had no significant effect on starch accumulation. However, we did find that there was significant difference between genotypes at ZT6, ($p<0.05$). Tukey's post hoc test showed that *HA-CDF1* starch levels were significantly less than *pSUC2::lncCDF1 #2.3* at ZT6 ($p<0.05$). We also found a significant difference in starch accumulation between genotypes at ZT16, using two way ANOVA ($p<0.05$). Tukey's post hoc test revealed that *HA-CDF1* starch levels were significantly lower than all other lines tested at ZT16 ($p<0.05$, Figure 12).

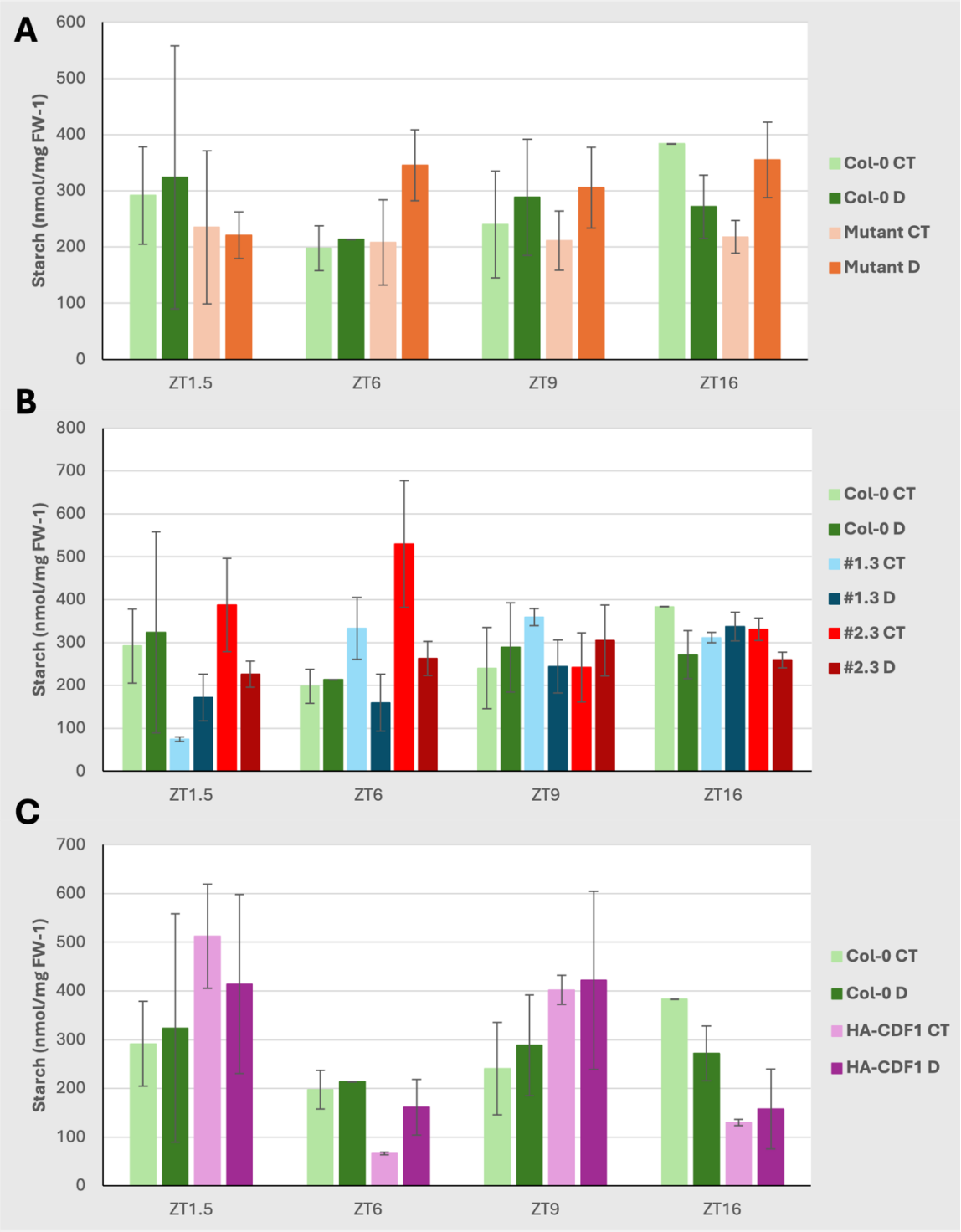


Figure 12: Starch accumulation (nmol/mg FW-1) in ZT1.5, ZT6, ZT9 and ZT16 in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) plants grown under control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor lines *(#1.3* and *#2.3*) grown under CT and D conditions; (C) wildtype (Col-0) and *HA-CDF1* overexpressor line grown under CT and D conditions. Bars represent mean starch concentration levels +/- standard deviation. Starch content was determined from two experimental replicates (n=2). Statistical analysis was performed independently at each time point using a two-way ANOVA followed by Tukey's multiple comparison test to assess the effects of genotype, treatment and the interaction between genotype and treatment. Significant differences are described in the results

3.4.2 Glucose

Examination of glucose levels from ZT1.5 to ZT16 showed that WT maintained relatively stable levels throughout the diurnal cycle, peaking at ZT6 and ZT16 in control plants (Figure 13A). Drought treated plants accumulated similar glucose levels to the control, with only a slight increase at ZT9. Similar to the starch levels, the *lnccdf1* mutant accumulated lower levels of glucose compared with WT under control conditions, except at ZT9. Drought conditions lead to higher glucose levels in all the timepoints except for ZT9, when they were comparable to the control plants (Figure 13A).

The overexpressor lines *pSUC2::lncCDF1 #1.3 and #2.3* grown under control conditions tended to accumulate more glucose than WT, except at ZT1.5 when *pSUC2::lncCDF1 #1.3* accumulates approx. 2x less (Figure 13B). Drought appeared to induce glucose accumulation in both overexpressor lines at ZT1.5 and ZT6.

Glucose levels in the *HA-CDF1* line were similar to WT under control conditions at ZT1.5, but in contrast to WT, drought plants had increased glucose levels (Figure 13C). At ZT6, glucose levels decreased in *HA-CDF1* for both treatments. Glucose accumulation increased again at ZT9 with higher levels in drought plants, before declining below WT values at ZT16 (Figure 13C).

A two-way ANOVA was performed to analyse the effect of genotype, treatment and the interaction between genotype and treatment has on glucose levels. We failed to detect a significant difference in the interaction between genotype and treatment. Statistical analysis revealed that glucose levels were significantly higher in drought conditions across all genotypes at ZT1.5 ($p<0.005$). The same test also found a significant difference between genotypes at ZT1.5 ($p<0.005$). Tukey's post hoc analysis showed that *pSUC2::lncCDF1 #2.3* has significantly higher levels of glucose than all other genotypes tested at ZT1.5 ($p<0.05$). Two-way ANOVA also revealed a significant difference in glucose accumulation between genotypes at ZT16 ($p<0.005$). Tukey's post hoc test revealed that *pSUC2::lncCDF1 #1.3* accumulated significantly more glucose than the *lnccdf1* mutant and *HA-CDF1* at ZT16 ($p<0.005$, Figure 13).

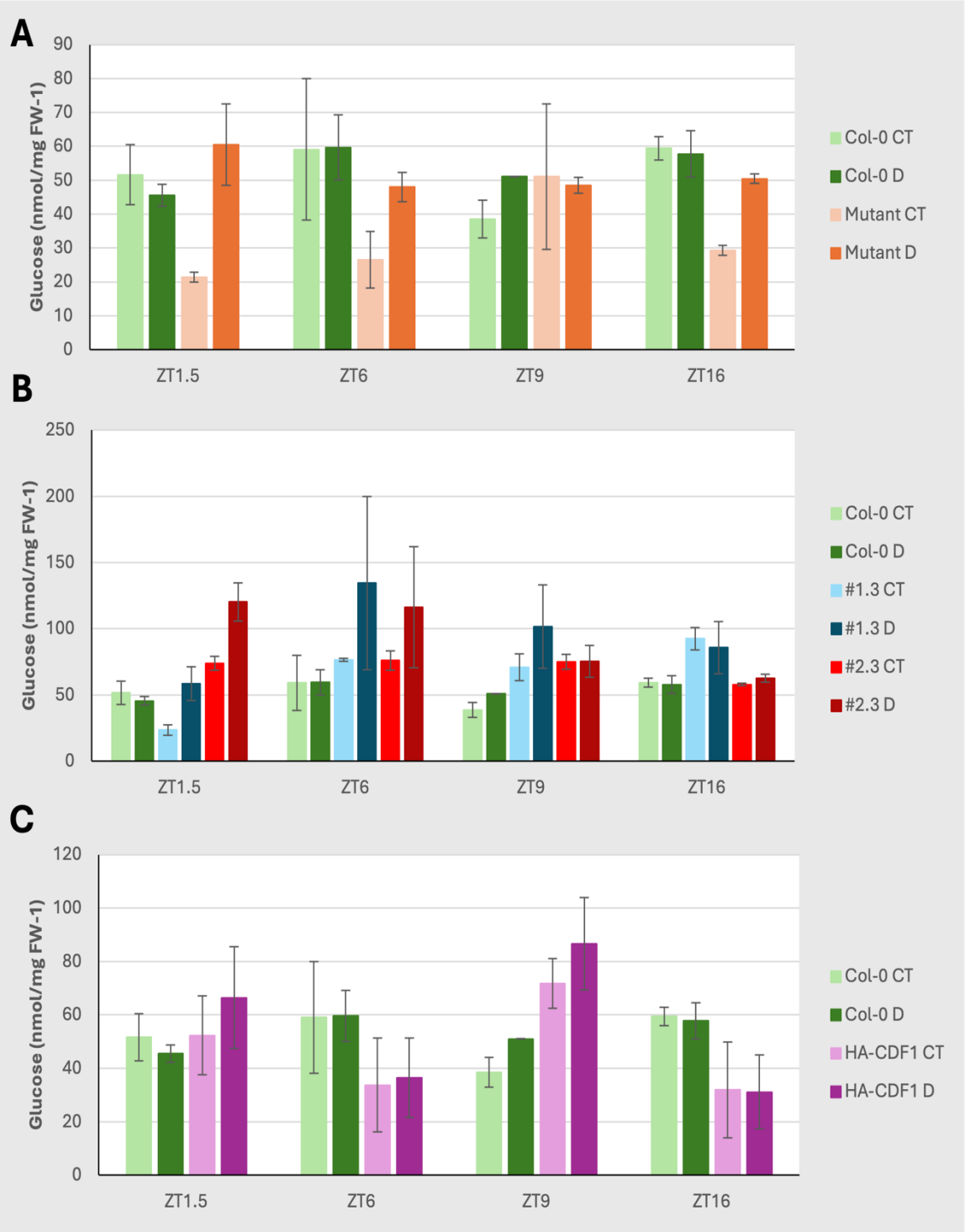


Figure 13: Glucose accumulation (nmol/mg FW-1) at ZT1.5, ZT6, ZT9 and ZT16 in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) under control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor lines *(#1.3* and *#2.3*) under CT and D conditions; (C) wildtype (Col-0) and *HA-CDF1* overexpressor line under CT and D conditions. Bars represent mean glucose concentration levels +/- standard deviation. Glucose content was determined from two experimental replicates (n=2). Statistical analysis was performed independently at each time point using a two-way ANOVA followed by Tukey's multiple comparison test to assess the effects of genotype, treatment and the interaction between genotype and treatment. Significant differences are described in the results section.

3.4.3 Fructose

Fructose accumulation was relatively stable throughout the day in WT plants grown under control conditions with a peak at ZT16 (Figure 14A). Drought appeared to have little effect on fructose levels, with slight increases observed at ZT6 and ZT9. The *lnccdf1* mutant control sample showed considerably less fructose accumulation than WT across all timepoints except ZT9. Interestingly, under drought conditions the *lnccdf1* mutant accumulated much higher levels of fructose throughout the entire time course (Figure 14A).

pSUC2::lncCDF1 #1.3 plants grown under control conditions displayed a similar trend to glucose levels, with low fructose levels at ZT1.5 but above those of WT and remained constant from ZT6 to ZT16 (Figure 14B). Drought treated plants appeared to accumulate more fructose across all timepoints except ZT16. The second overexpressor line *lncCDF1 #2.3* maintained relatively higher levels of fructose compared to WT from ZT1.5 to ZT9 both under both control and drought conditions. Fructose levels were similar to WT at ZT16, with no major differences in drought plants (Figure 14B).

Fructose levels in *HA-CDF1* control plants were generally low compared with WT, although fructose levels peaked higher than WT at ZT9 (Figure 14C). Drought appeared to have a strong effect on fructose accumulation at ZT1.5, but no major changes at the other timepoints.

Two-way ANOVA was carried out to test significant differences in genotype, treatment and the interaction between genotype and treatment. Analysis revealed no significant differences in the interaction between genotype and treatment. However, we did find that fructose levels are significantly higher in drought plants across all genotypes at ZT1.5 ($p<0.05$). Using the same test we also found a significant difference in fructose levels between genotypes at ZT6 ($p<0.05$). Tukey's post hoc analysis revealed that *pSUC2::lncCDF1 #1.3* had significantly higher levels of fructose than *HA-CDF1* at ZT6. Furthermore, a two-way ANOVA for ZT16 revealed a statistically significant difference in fructose levels between genotypes ($p<0.005$). Tukey's post hoc test revealed that *pSUC2::lncCDF1 #1.3* had significantly higher fructose levels than the *lnccdf1* mutant and *HA-CDF1* at ZT16 (Figure 14).

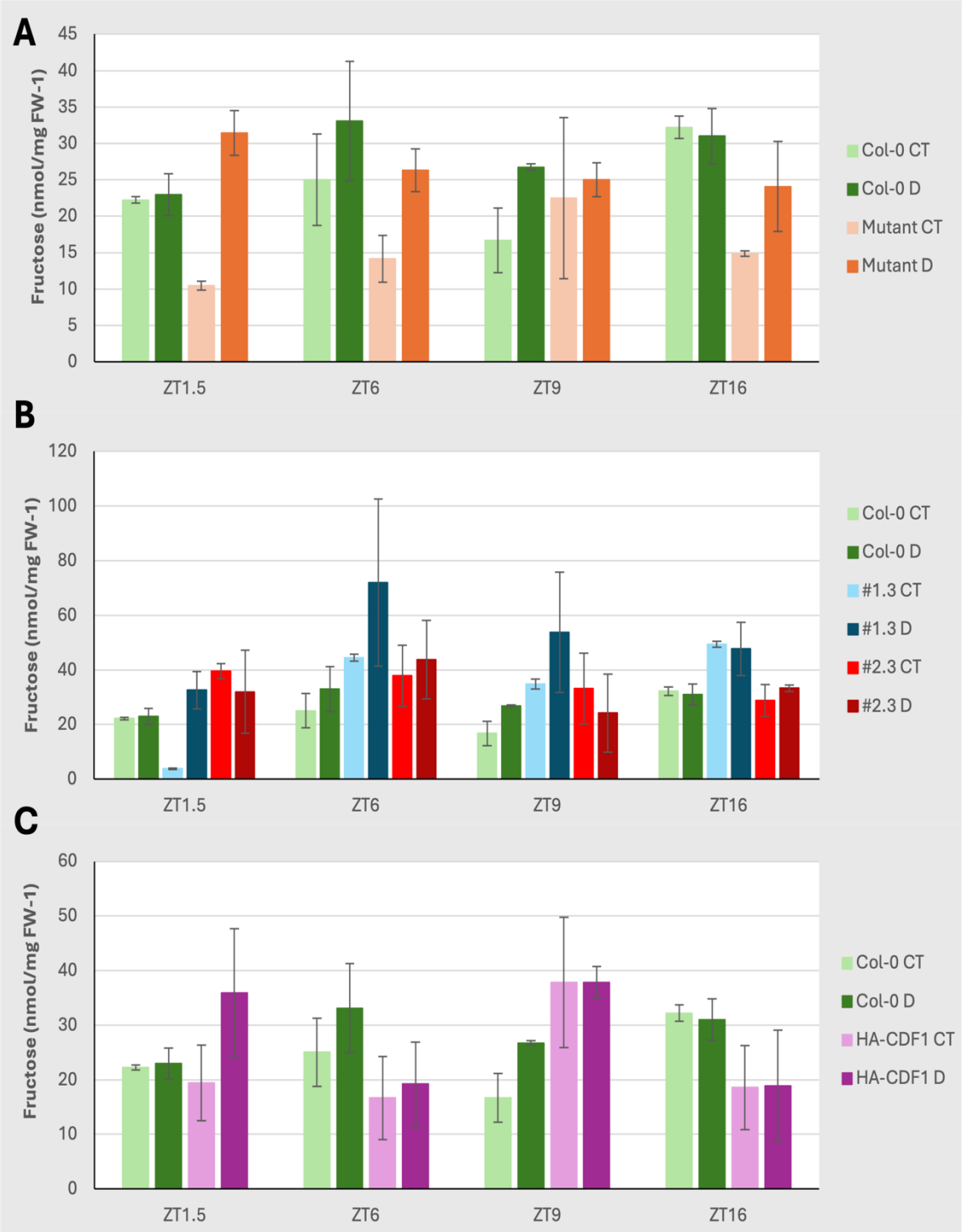


Figure 14: Fructose accumulation (nmol/mg FW-1) at ZT1.5, ZT6, ZT9 and ZT16 in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) under control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2::lncCDF1* overexpressor lines *(#1.3* and *#2.3*) under CT and D conditions; (C) wildtype (Col-0)and *HA-CDF1* overexpressor line under CT and D conditions. Bars represent mean fructose concentration levels +/- standard deviation. Starch content was determined from two experimental replicates (n=2). Statistical analysis was performed independently at each time point using a two-way ANOVA followed by Tukey's multiple comparison test to assess the effects of genotype, treatment and the interaction between genotype and treatment. Significant differences are described in the results section.

3.4.4 Sucrose

The analysis of sucrose levels showed a gradual increase of sucrose throughout the day in WT plants, representing diurnal accumulation under control conditions (Figure 15A). Drought had contrasting effects on sucrose levels, since it decreased at ZT1.5 and ZT16, but increased at ZT6 and ZT9. The *lnccdf1* mutant had lower levels of sucrose than the WT, with the exception of ZT9. There was a clear increase in sucrose accumulation under drought conditions across the entire time course (Figure 15A).

The overexpressor lines exhibited different sucrose profiles. *pSUC2::lncCDF1 #1.3* had low sucrose levels at ZT1.5 but it was greater than WT at the remaining timepoints (Figure 15B). Drought plants accumulated more sucrose compared to WT across all timepoints. In contrast, *pSUC2::lncCDF1 #2.3* had elevated and gradually increasing sucrose levels as the day progressed, peaking at ZT16. Drought treated plants tended to have a varied response in sucrose accumulation, increasing at ZT6 and ZT16 and decreasing at ZT1.5 and ZT9 (Figure 15B).

Sucrose levels are higher in *HA-CDF1* compared with WT grown under control conditions, except at ZT16. Drought did not appear to have a major effect on sucrose levels in *HA-CDF1* plants from ZT1.5 to ZT6, with only slight increases observed at ZT9 (Figure 15C).

A two-way ANOVA was conducted to assess the effects of genotype, treatment and their interaction on sucrose levels. The analysis indicated that treatment and the interaction between genotype and treatment had no significant effect. Statistical analysis using two-way ANOVA revealed that there was a significant different in sucrose levels between genotypes at ZT6 ($p<0.005$). Tukey's post hoc analysis showed that *pSUC2::lncCDF1 #1.3* had significantly higher sucrose levels than all other genotypes at ZT6 ($p<0.05$). Using the same test, we also found a significant difference in starch levels between genotypes at ZT16 ($p<0.05$). Tukey's post hoc test revealed that *pSUC2::lncCDF1 #1.3* had significantly more sucrose than *lnccdf1* mutant and *HA-CDF1* at ZT16 ($p<0.05$, Figure 15).

These results show that, while time of day trends of starch metabolism remained broadly consistent, the impact and direction of drought responses showed substantial differences, especially in *pSUC2::lncCDF1 #2.*3 and *HA-CDF1* plants. Furthermore, this data highlights the effect *lncCDF1* expression has on diurnal sugar metabolism in *A. thaliana*, suggesting a functional link between *lncCDF1* and sugar homeostasis under drought conditions.

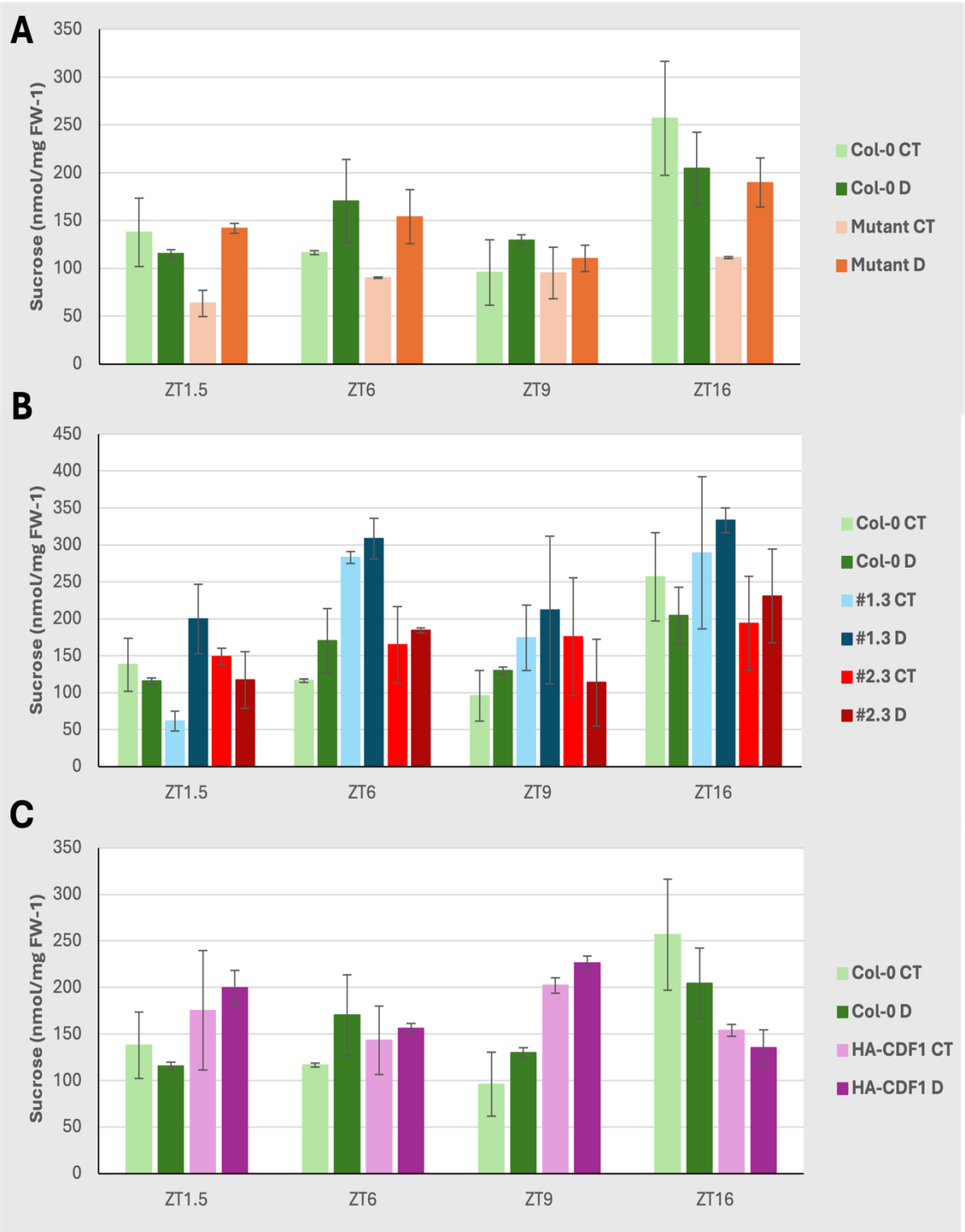


Figure 15: Sucrose accumulation (nmol/mg FW-1) at ZT1.5, ZT6, ZT9 and ZT16 in (A) wildtype (Col-0) and *lnccdf1 mutant #08880-10* (Mutant) under control (CT) and drought (D) conditions; (B) wildtype (Col-0) and *pSUC2lncCDF1* overexpressor lines *(#1.3* and *#2.3*) under CT and D conditions; (C) wildtype (Col-0) and *HA-CDF1* overexpressor line under CT and D conditions. Bars represent mean sucrose concentration levels +/- standard deviation. Starch content was determined from two experimental replicates (n=2). Statistical analysis was performed independently at each time point using a two-way ANOVA followed by Tukey's multiple comparison test to assess the effects of genotype, treatment and the interaction between genotype and treatment. Significant differences are described in the results section.

4.0 Discussion

4.1 *LncCDF1* functions as regulator of photoperiodic flowering under drought conditions

The regulation of flowering time is an essential process for plant reproduction and survival, with continued research adding to the complex genetic architecture of flowering plants. Recent studies suggest that the NAT pair *lncCDF1/CDF1* like other *CDF* related NAT pairs such as *CDF5/FLORE* play a potential regulatory role in photoperiodic flowering, as well as abiotic stress responses (Henriques *et al.,* 2017). This study aimed to expand on the current knowledge of *lncCDF1* biological function and specifically characterize its role in drought responses. In order to do so, we started by examining flowering time of different *A. thaliana lncCDF1* lines (mutant and overexpressors) grown under control and drought conditions.

It is well described that CDF1 represses the expression of key floral activator genes such as *CO* and *FT* in *A. thaliana* through a transcriptional complex between CDFs and TOPLESS (*TPL*) (Goralgia *et al.,* 2017). Furthermore, unpublished data identified an upregulation of *CDF1* and *lncCDF1* transcription in response to drought. It has been reported that in potato the overexpression of the lncRNA, *StFLORE* improved plant health under drought stress through influencing stomatal growth and diurnal opening (Gonzales *et al.,* 2021). The counterpart of this lncRNA, *StCDF1* is known to regulate tuberization and when down regulated it increases the plants tolerance to drought (Gonzales *et al.,* 2021). As tuberization and flowering time undergo similar photoperiod regulation, *lncCDF1* may act as a key regulatory link integrating photoperiod signalling with drought stress responses. Our results indicate that the overexpression of *lncCDF1* promotes a slight early flowering response, especially in the days to flowering although these differences are not statistically significant. Whereas the misexpression of *lncCDF1* in the *lnccdf1 salk-mutant #08880-10* did not result in significantly later flowering than the WT but produced significantly more rosette leaves. Moreover, the overexpression of *CDF1* has been associated with a late flowering phenotype as previously described using the transgenic line *HA-CDF1* (Imaizumi *et al.,* 2005). However, when grown under control conditions this *HA-CDF1* line presented a similar flowering time to that of

wildtype, but when exposed to drought stress these plants had a significant higher number of rosettes leaves when they flowered (Figure 5). To validate our experimental conditions we tested the *cdf-quadruple* mutant, which displays photoperiod-insensitive early flowering, and as expected, we observed significant early flowering in both control and drought conditions (Fornara *et al.,* 2009).

Overall, this data suggests that *lncCDF1* is involved in the regulation of photoperiodic flowering by fine-tuning the process rather than mediating major developmental transitions. As *CDF1* represses the expression of *CO*, the upregulation of *lncCDF1* could modulate the inhibitory effects of *CDF1* in promoting the transition to flowering (Praena *et al.,* 2022). This coincides with studies exploring *CDF5* and its antisense transcript *FLORE* which display a similar regulatory process to promote flowering under LD conditions (Henriques *et al.,* 2017). It is also reported that *FLORE* could act in trans on *CDF1*, highlighting the complex nature of CDF regulated photoperiodic flowering (Henriques *et al.,* 2017). As noted previously, unpublished data from Dr. Henriques showed that *lnccdf1* mutant lines displayed greater tolerance to drought conditions, suggesting that *lncCDF1*'s regulatory influence is more important under drought stress rather than serving as a major contributor to photoperiodic flowering under control well-watered conditions.

4.1 Drought-induced modulation of *lncCDF1* expression

Previous unpublished data from Dr. Henriques' research group showed that *lncCDF1/CDF1* do not follow the same antiphasic pattern as *CDF5/FLORE* but display a phase shift as we observe a delay in *lncCDF1* peak expression when compared to *CDF1* (Henriques *et al.,* 2017). In *A. thaliana* plants grown under long day conditions *CDF1* will peak at early morning (ZT0-ZT4) and its trough from ZT8-ZT20; whereas *lncCDF1* shows a phase shift peaking slightly later at ZT6 and will be minimal at ZT18 (Figure 16).

It is also reported that both *lncCDF1* and *CDF1* are upregulated in *A. thaliana* seedlings grown under drought conditions (Van Veen, 2021). This study confirmed that altering *lncCDF1* expression results in differential *CDF1* transcript levels, but did not find that drought stress increased *lncCDF1* and *CDF1* expression levels in all time points tested.

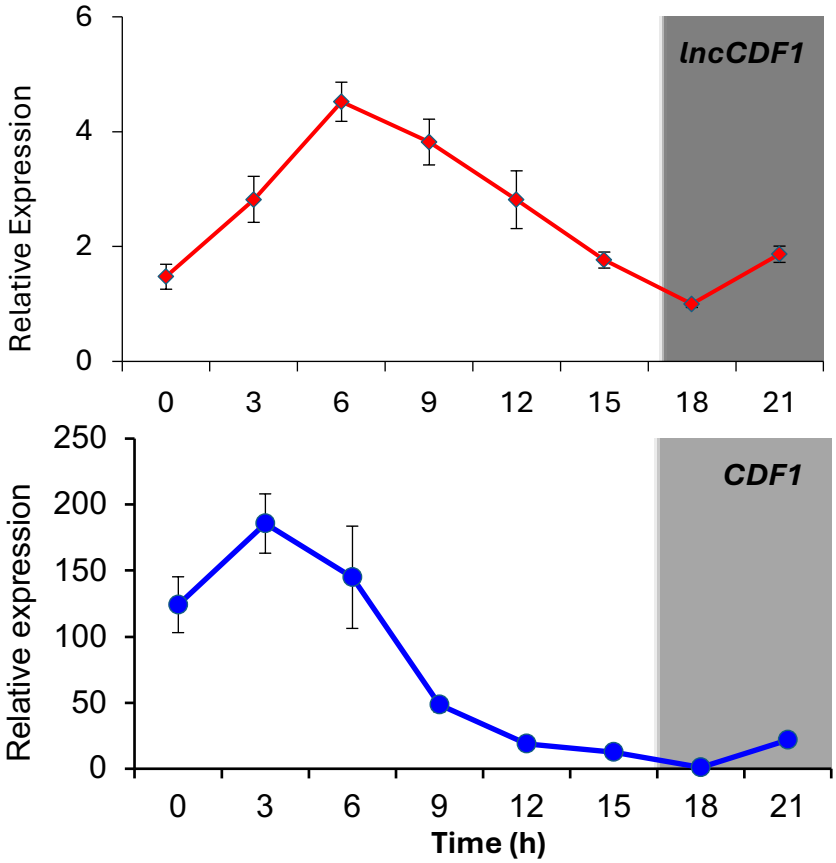


Figure 16: Unpublished data provided by Dr. Henriques relating to the relative expression patterns of *lncCDF1* (top) and *CDF1* (bottom) in *A. thaliana* grown under long day conditions.

Further studies examining *lncCDF1* expression of developed plants in response to ABA failed to report a consistent expression pattern following ABA exposure (Van Veen, 2021). Here we examined the effect of drought stress in soil plugs grown seedlings 50% water content, to determine *lncCDF1* expression responses in different transgenic lines (de Ollas *et al.,* 2019). Similar to previous results in our laboratory, wildtype plants exhibited an upregulation of *lncCDF1* levels under drought conditions especially at ZT1.5, while the *lncCDF1* transgenic lines showed differential responses to drought. Both *lnccdf1* mutant and overexpressor line *pSUC2::lncCDF1#1.3* experienced a consistent down regulation of *lncCDF1* expression, although *lncCDF1* transcript levels were much greater in the overexpressor line. In contrast, the second overexpressing line *pSUC2::lncCDF1 #2.3* displayed a clear upregulation of *lncCDF1* expression under drought conditions. Furthermore, we observed no clear drought responsive pattern in the *HA-CDF1* mutant from ZT1.5-ZT6, but there was a strong upregulation of *CDF1* at ZT9.

CDF1 expression typically peaks at early morning and decreases for the remainder of the day when plants are grown under LD conditions (Martín *et al.,* 2020). This coincides with our data, as we observed an early morning peak for *CDF1* and then a decrease in expression for the remaining timepoints in wildtype plants grown under control conditions. Previous studies by Dr. Henriques research group showed that *CDF1* expression increased under drought conditions, but seedlings were grown in pots without watering for 10 days, whereas in this experimental set-up wildtype seedlings were kept at 50% of water content for 3 days (de Ollas *et al*., 2019). Although in this study we sampled different timepoints, *CDF1* expression was only upregulated by drought at one instance (ZT6). The *lnccdf1* mutant lines exhibited unique *CDF1* expression patterns with differential responses to drought, with only the weak overexpressor line *pSUC2::lncCDF1 #2.3* displaying a clear upregulation of *CDF1* transcript levels under drought conditions. As expected the transgenic overexpressor line *HA-CDF1* showed high *CDF1* expression levels, while drought responses did not follow a clear pattern, which suggest that the overexpression of *CDF1* may override the effects of the drought stress. Previous studies suggest that *CDF* genes are regulated by abiotic stresses where plants overexpressing CDFs show improved tolerance to abiotic stress (Docimo *et al.,* 2024). Examples include tomato plants over-expressing *CDF3* genes from *Arabidopsis* showed increased biomass and yield under salinity stress conditions (Renau-Morata *et al.,* 2017).

Such results suggest that *lncCDF1* and *CDF1* expression is modulated by drought stress, and these responses get altered as a result of different basal levels of both transcripts. Although it is clear that expression levels change under drought conditions, this study only analysed plants from one replicate. Further replication would be required to confirm drought responsive expression patterns. It is also important to consider whether *lncCDF1* and *CDF1* expression responds proportionally to increasing stress severity or is triggered after a threshold. Therefore further research should investigate expression responses to different drought stress levels.

4.2 Exploring *lncCDF1* targets in response to drought

Previous RNA-seq analysis identified five genes that exhibited the greatest changes in expression in response to drought, these include; *QUA QUINE STARCH (QQS)*, *SUCROSE-PROTON SYMPORTER 7 (SUC7)*, *OSMOTIN 34 (OSM34)*, *EXTENSIN 1 (EXT1)*, and

MOTHER OF FT AND TFL (MFT). Among the genes tested, *QQS* which has reported to promote starch metabolism, is the only gene to show higher expression in the wildtype compared with all other genotypes. Interestingly, *QQS* expression is down regulated under drought conditions suggesting some inhibitory effect. Similarly, the mutation in *lncCDF1* promoter inhibits *QQS* accumulation, with varied expression responses to drought. Studies reported that *QQS* promotes starch degradation for the opening of stomata (Ding *et al.,* 2014). Recent studies on bioengineered cassava overexpressing *QQS* from *A. thaliana* improved starch and protein content of plants (Hankoua *et al.,* 2025). In potato, plants expressing *QQS* and overexpressing Nuclear Factor Y subunit C4 (*NF-YC4*) demonstrated increased protein and reduced starch content without compromising tuber yield and quality (Tanvir *et al.,* 2022). The upregulated levels of *QQS* expression at ZT9 in the *lnccdf1* mutant under drought conditions may explain their improved drought tolerance, as plants promote stomatal opening to accommodate photosynthesis later in the day. Further research investigating stomatal opening would be required to validate this drought response.

SUC7 encodes a sucrose proton symporter described to increase its expression levels under drought conditions in wildtype plants, which coincides with the results we observed (Ahmad *et al.,* 2021). Interestingly, *SUC7* does not function as an active sucrose transporter like other *SUC* genes, but instead it may have a regulatory function such as a sucrose sensor (Rottmann *et al.,* 2018). As drought stress activates sugar signalling pathways, SUC7 may improve sucrose sensing to help optimise source-to-sink distribution of sugars (Kaur *et al.,* 2021). The upregulation of *SUC7* expression in the *lnccdf1* mutant suggest a potential association between *lncCDF1* and sugar signalling pathways, adding an additional regulatory layer to improve drought tolerance.

We also observed an upregulation of *OSM34* in both *lnccdf1* mutant and *pSUC2::lncCDF1 #1.3* overexpressor lines. Studies have reported that *OSM34* functions in the ABA signalling pathway as a positive regulator of ABA responses by increasing proline accumulation which can function as ROS scavengers (Park and Kim, 2021). *CDF1* and *lncCDF1* have recently been shown to respond to ABA especially in the early stages of seed germination (Van Veen, 2021). Our data suggests that a component of ABA signalling such as *OSM34* that functions as a regulator of drought responses could be modulated by *lncCDF1* to enhance plant tolerance to drought. To more clearly define the functional relationship between *lncCDF1* and this ABA response regulator, *OSM34* expression could be systematically assessed in *lncCDF1* transgenic lines under controlled ABA application and progressive drought stress.

Similarly to the previous genes identified in our RNA-Seq experiment, *MFT* also plays a role in ABA responses, and promotes embryo growth (Xi *et al.,* 2010). *MFT* also exhibits *FT*-like properties and modulates flowering time, where its overexpression accelerates flowering (Yoo *et al.,* 2004). Interestingly, an *MFT* homolog from orchid *DnMFT* resulted in delayed flowering when constitutively expressed in *A. thaliana* (Li *et al.,* 2012). As *MFT* sequence is similar to *FT* and *TFL1* it is difficult to predict its function. However, the *MFT* homolog *MiMFT* identified in mango improved salt and drought tolerance when overexpressed in *A.* thaliana as well as being involved in seed germination (Lu *et al.,* 2022).

Notably the *lnccdf1* mutant did not appear to flower earlier than wildtype but showed an upregulation of *MFT* expression under drought conditions at early morning, although we failed to detected *MFT* at ZT9. Moreover, the weaker *lncCDF1* over-expressing line *pSUC2::lncCDF1 #2.3* had a much greater accumulation of *MFT* under drought conditions and did show an early flowering phenotype. Our results reveal that *MFT* expression is affected by *lncCDF1* with a greater influence in effect under drought conditions.

The remaining candidate *EXT1* had previously been reported to function in response to biotic stresses and physical wounds. When overexpressed, *EXT1* alters stem morphology and limits pathogen invasiveness by contributing to cell wall assembly and strengthening (Roberts *et al.,* 2012). Studies also suggest that extensins are often induced by abiotic stresses such as drought to improve water loss (Merkouropoulos and Shirsat, 2003, Kalve *et al.,* 2014). Our data shows a strong drought induced accumulation of *EXT1* in WT plants, whereas *lncCDF1* transgenic lines exhibited varied response patterns suggesting a disruption to regulatory networks. These findings highlight the regulatory network surrounding *lncCDF1* and the complex pathways involved in drought stress responses.

Collectively, the differential expression of these candidate genes suggests that *lncCDF1* influences drought responses through multiple interconnected pathways, including carbohydrate metabolism. In particular, the altered expression of *QQS* and *SUC7* provides a potential mechanistic explanation for the changes in starch and soluble sugar accumulation observed in the *lncCDF1/CD*F1 transgenic lines as these genes have established roles in carbon allocation and sugar signalling (O'Connor *et* al., 2018, Rottmann *et al*., 2018). These transcriptional changes supported by the altered patterns of starch and soluble sugar accumulation suggests that *lncCDF1* may regulate not only genes involved in carbohydrate metabolism but also downstream metabolic responses under drought conditions. This

relationship is consistent with previous studies showing that drought stress induces extensive reprogramming of carbohydrate metabolism, characterised by starch degradation and the accumulation of soluble sugars to function as osmoprotectants and signalling molecules (Ahmad *et al*., 2020, Afzal *et al*., 2021). In addition to functioning as osmoprotectants, soluble sugars act as key signalling molecules that modulate stress responsive gene expression through extensive crosstalk with ABA signalling (Krasensky and Jonak, 2012, Xue *et al*., 2025). The enhanced accumulation of these soluble sugar in the *lncCDF1* overexpression lines under drought conditions complements the RT-qPCR data, indicating that *lncCDF1* may regulate drought adaption by integrating transcriptional responses with carbon partitioning.

4.3 *lncCDF1* effects diurnal starch and soluble sugar metabolism

Following the characterisation of multiple drought responsive genes in *lncCDF1* transgenic lines, we observed changes of expression in starch and soluble sugar related genes. Therefore, we analysed starch and soluble sugar content of all these lines grown under control and drought conditions. It is well understood that in plants, photosynthesis produces sugars during the day, which serves as both an immediate energy source and a substate for metabolic processes (Du *et al.,* 2000). Assimilates are then converted to starch and stored, allowing plants to maintain energy supply (Arias *et al.,* 2014). This photosynthetic partitioning is under circadian regulation to fine tune starch metabolism and ensure survival in the subsequent night when the photosynthetic rate is low. This will ensure that stored starch is degraded and exported to sink organs (Zeeman *et al.,* 2006).

Typically, starch levels are highest just before the night, which we did not observe in the *lncCDF1* transgenic lines under control conditions. Interestingly, the *lnccdf1* mutant had greater accumulation of starch under drought compared with wildtype grown under similar conditions at that time point. Starch content was higher at early morning timepoints in both *pSUC2::lncCDF1 #2.3* and *HA-CDF1* control plants, suggesting that the overexpression of *lncCDF1* and *CDF1* disrupts starch metabolism throughout the night and the stored starch was not totally used. However, before the night period, starch levels are much lower in *HA-CDF1*,

indicating that the over expression of *CDF1* alters diurnal starch accumulation. Moreover, when *lncCDF1* transgenic lines were grown under drought conditions, starch accumulation appeared more stable throughout the day. Studies on drought treated rice plants showed a similar decrease in starch content in response to drought to what we observed in the wildtype, which is opposite to what we see in the *lnccdf1* mutant (Kim *et al.,* 2017). This suggests that *lncCDF1* could modulate starch accumulation under drought conditions.

Starch can also be degraded to glucose and glucose-1-phosphate during the day in periods of drought, which allows the synthesis of sucrose at the end of the day to provide crucial protection against oxidative stress (Ramel *et al.,* 20217). This supports our data as *lnccdf1* mutant and *lncCDF1* overexpressors increased sucrose levels at ZT16 similar to WT. We see a difference at ZT6 for all soluble sugars as *pSUC2::lncCDF*1 consistently shows higher sugar levels under drought conditions. Similarly, the *lnccdf1* mutant showed increased accumulation of soluble sugars under drought stress, which may explain its improved tolerance to drought. Although few studies explore the link between carbohydrate diurnal rhythm and drought stress it is difficult to infer this with certainty. Previous studied reported that soluble sugar accumulation increased with drought severity (Sperdouli and Moustakas, 2012). We observed a similar response with an increase of glucose and fructose accumulation under drought conditions for all transgenic lines, whereas wildtype did not show increased sugar levels under drought stress across all timepoints.

This data indicates that *lncCDF1* adds an additional regulatory layer to find tune drought responses through a complex network of pathways. Although further research examining these transgenic lines under different stress severities might reveal a regulation pattern. These results shed light on the importance of lncRNA-mediated regulation of drought responses and highlight the need for more mechanistic studies to uncover how *lncCDF1* interacts with drought signalling pathways.

5.0 Conclusion

This thesis aimed to elucidate the regulation properties of the *NAT* pair *lncCDF1/CDF1* under drought conditions in *A. thaliana*. By assessing this *NAT* pair we sought to uncover whether *lncCDF1* functions as a regulator of photoperiodic flowering. Our results suggest that *lncCDF1* influences flowering time, where its overexpression can promote early flowering under both control and drought conditions and a knockdown mutation resulted in a late flowering phenotype. Furthermore, our findings support the role of *lncCDF1* in regulating drought responsive genes in *A. thaliana* for improved drought tolerance, as qPCR data revealed unique expression profiles of these genes in *lncCDF1* transgenic lines, both mutants and overexpressors. Finally, the determination of starch and soluble sugars provide an insight into the regulatory effects *lncCDF1* has on diurnal sugar metabolism under drought conditions. In particular, the *lnccdf1* mutant accumulates low levels of soluble sugars under control conditions, but under drought stress can accumulate up to 3x more soluble sugars. The findings described in this study help to further understand the vast physiological and molecular processes influenced by lncRNAs.

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