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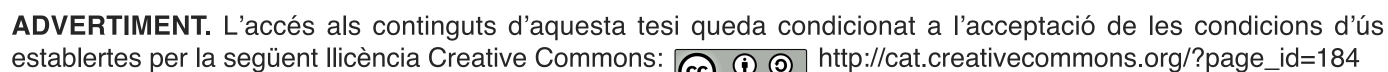
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

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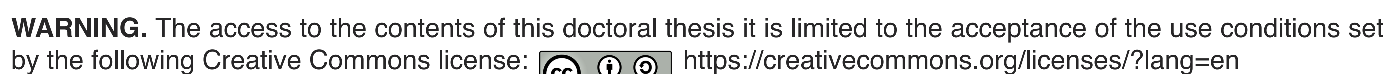
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

PhD Thesis

Elucidating TEM and MYC roles in *Arabidopsis thaliana* floral repression

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Programa de Doctorat en Biologia i Biotecnologia Vegetal

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Elucidating TEM and MYC roles in *Arabidopsis thaliana* floral repression

Dissertation presented by Poonam Nebhnani for the degree of Doctor of Plant Biology and Biotechnology at the Autonomous University of Barcelona. This work was done in the Centre for Research in Agricultural Genomics (CRAG).

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बहुत-बहुत शुक्रिया, मम्मी और लालू भालू।

ABSTRACT

The period in which a plant transitions from juvenile to adult phase and therefore flowering can be induced, is a crucial determining factor for reproductive success. Plants initiate this process based on various internal and external signals. After perception of these signals a complex gene regulatory network activates or represses floral induction.

TEMPRANILLO (TEM) genes are transcription factors identified by our lab in *Arabidopsis thaliana.* TEM proteins are involved in delay of flowering by repression of *FLOWERING LOCUS T* (*FT*, a signal for flowering initiation) by binding to a region near its transcription start site and blocking transcription. Our main goal has been to dissect the mechanisms of TEM activity in controlling flowering time and understanding how these proteins reprogram plant development for environmental adaptation.

Yeast 2-hybrid experiments revealed that basic Helix Loop Helix transcription factors MYC2 and MYC4 are TEM interactors. MYC2 also binds to the *FT* promoter (same region where TEM binds) during jasmonic acid mediated flowering inhibition. Other related and similar functions between TEM and MYC led us to hypothesize that these transcription factors may work as a complex in repressing *FT*, hence hindering flowering. To follow this train of thought, we designed chromatin immunoprecipitation (ChIP) experiments to check whether these factors bound to their target genes independently or if the presence of the other made a difference in their action. By doing this, our results suggested that these transcription factors possibly require the presence of the other to bind to target genes.

MYC and TEM interact with a few other factors, including TOPLESS also suggested that the complex might recruit histone modification enzymes to suppress expression of target genes. Therefore, in order to unravel this, we have performed ChIP-qPCR studies. These results could not prove any definite connection between the phenotype of mutant plants with the levels of histone acetylation or methylation marks found in the samples.

The upregulation of *FT* transcription depends largely on forming a loop which brings *FT* activator CONSTANS closer to its regulatory element and causes transcription. To check if TEM disrupted the formation of this loop, we performed nucleosome occupancy studies. Although, we did not find differences in samples collected under long day conditions, under short days the earliest flowering mutant had a much more open chromatin which could

indicate that these transcription factors somehow play a role in modulating chromatin configuration on the *FT* locus.

RESUMEN

El período en el que una planta pasa de la fase juvenil a la adulta y, por lo tanto, se puede inducir la floración, es un factor determinante para su éxito reproductivo. Las plantas inician este proceso basándose en varias señales internas y externas. Después de la percepción de estas señales, una compleja red reguladora de genes activa o reprime la inducción floral.

Los genes *TEMPRANILLO* (*TEM*) son factores de transcripción identificados por nuestro laboratorio en *Arabidopsis thaliana*. Las proteínas TEM están involucradas en el retraso de la floración mediante la represión de *FLOWERING LOCUS T* (*FT*, una señal para el inicio de la floración) uniéndose a una región cercana a su sitio de inicio de transcripción y bloqueando la transcripción. Nuestro objetivo principal ha sido diseccionar los mecanismos de la actividad TEM en el control del tiempo de floración y comprender cómo estas proteínas reprograman el desarrollo de las plantas para adaptarse al medio ambiente.

Los experimentos con 2 híbridos de levadura revelaron que los factores de transcripción Helix Loop Helix básicos MYC2 y MYC4 interactúan con TEM. MYC2 también se une al promotor *FT* (la misma región donde se une TEM) en la represión de la floración mediada por ácido jasmónico. Otras funciones similares entre TEM y MYC nos llevaron a plantear la hipótesis de que estos factores de transcripción podrían funcionar como un complejo proteico en la represión de *FT*, lo que retrasaría la floración. Siguiendo esta línea de pensamiento, diseñamos experimentos de inmunoprecipitación de cromatina (ChIP) para verificar si estos factores se unían a sus genes diana de forma independiente o si la presencia del otro marcaba una diferencia en su acción. Al hacer esto, nuestros resultados sugirieron que estos factores de transcripción posiblemente requieran la presencia del otro para unirse a los genes diana.

MYC y TEM interactúan con otros factores, incluido TOPLESS lo que sugería que el complejo podría reclutar enzimas de modificación de histonas para suprimir la expresión de genes diana. Por lo tanto, para desentrañar esto, hemos realizado estudios de ChIP-qPCR. Sin embargo, estos resultados no pudieron probar ninguna conexión definitiva entre el

fenotipo de las plantas mutantes con los niveles de acetilación de histonas o marcas de metilación encontradas en las muestras.

La regulación positiva de la transcripción de *FT* depende en gran medida de la formación de un bucle que acerque al activador de *FT* CONSTANS a su elemento regulador e induzca la transcripción. Para verificar si TEM impide la formación de este bucle, realizamos estudios de ocupación de nucleosomas. Aunque no encontramos diferencias en las muestras recogidas en condiciones de día largo, en día corto el mutante de floración más temprano tenía una cromatina mucho más abierta, lo que podría indicar que estos factores de transcripción de alguna manera juegan un papel en la modulación de la configuración de la cromatina en el locus *FT*.

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List of Abbreviations

5'UTR	5' Untranslated Region
AP2	APETALA2
bHLH	basic Helix Loop Helix
BiFC	Bimolecular Florescence Complementation
BRD	B3 Repression Domain
CaRAV	*Capsicum annuum* RAV
CDFs	CYCLING DOF FACTORS
ChIP	Chromatin Immunoprecipitation
CLF	CURLY LEAF
CO	CONSTANS
CoIP	Co-immunoprecipitation
CORE 1&2	CO Responsive elements 1&2
CsRAV1	*Castanea Sativa RAV1*
EB	Extraction Buffer
ELF3	EARLY FLOWERING 3
FAIRE	Formaldehyde Associated Isolation of Regulatory Elements
FKF1	FLAVIN-BINDING, KELCH REPEAT, F-BOX1
FL	*FLOWERING LOCUS C*
FLD	*FLOWERING LOCUS D*
FLK	*FLOWERING LOCUS K*
FRI	*FRIGIDA*
FT	*Flowering Locus T*
GA	Gibberellic Acid
GA20OX2	*Gibberellic Acid 20 oxidase 2*
GA3ox1	*Gibberellic Acid 3 oxidase 1*
GA3ox2	*Gibberellic Acid 3 oxidase 2*
GI	GIGANTEA
GmRAV	*Glycine max RAV*

H3K27me3	Trimethylation of histone H3 lysine 27
HATs	Histone Acetyl Transferases
HDACs	Histone Deacetylases
HFD	Histone Fold Domain
JA	Jasmonic Acid
JAZ	JASMONATE-ZIM DOMAIN
LD	Long Day
LFY	*LEAFY*
LHP1	LIKE HETEROCHROMATIN PROTEIN1
LKP2	LOV KELCH PROTEIN2
LHY	*LATE ELONGATED HYPOCOTYL*
MeRAV	*Manihot esculenta RAV*
miR156	micro-RNA 156
NDRs	Nucleosome Depleted Regions
NF-Y	Nuclear Factory Y
NLB	Nuclei Lysis Buffer
PcG	Polycomb Group proteins
PbRAV	*Pyrus bretschneideri RAV*
PHYA	Phytochrome A
PK	PICKLE
PRC2	Polycomb Repressive Complex 2
PtRAV	*Poplus trichocarpa RAV*
RAV	Related to ABI3/VP1
RAV1L	RAV1 Like
REF6	RELATIVE OF EARLY FLOWERING 6
RT	Room Temperature
SAM	Shoot Apical Meristem
SD	Short Says
SDG	SET Domain Group

SlRAV	*Solanum lycopersicon RAV*
SMZ	*SCHLAFMÜTZE*
SNZ	*SCHNARCHZAPFEN*
SOC1	*SUPRESSOR OF OVEREXPRESSOR OF CONSTANS 1*
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*
SRR1	SENSITIVITY TO RED LIGT REDUCED1
SVP	VEGETATIVE PHASE
TAD	Transcriptional Activation Domain
TEM	*TEMPRANILLO*
TEM1	*TEMPRANILLO 1*
TEM2	*TEMPRANILLO 2*
TFs	Transcription Factors
TOE1	TARGET OF EAT 1
TOE2	TARGET OF EAT 2
TOE3	TARGET OF EAT 3
TPL	TOPLESS
TPR	TPL-related
TSF	*TWIN SISTER OF FT*
TSS	Transcription Start Site
Y2H	Yeast 2-hybrid
WT	Wild Type
ZTL	ZEITLUPE

1. INTRODUCTION

1.1 FLOWERING

Flowering is a process in which a plant forms flowers, which ultimately turn into fruits. Angiosperms, or flowering plants, evolved about 125-100 million years ago. It is theorised that this evolution, besides protecting ovules, helped attract pollinators and make cross-pollination easier and faster (Openstax Biology book). Flowering occurs when a plant converts its vegetative meristem to the floral meristem. Every angiosperm plant species, whether annual or perennial, from the temperate or tropical region, undergoes flowering. Since plants are sessile organisms, they have developed robust pathways which help them navigate growth and reproduction in favourable environmental conditions. The period in which a plant transitions from juvenile to adult phase and therefore flowering can be induced, is a crucial determining factor in its degree of reproductive success (Purugganan and Fuller, 2009). It is a crucial process because formation of flowers predates seed-set. Reproductive success is measured by effective seed-set, the higher the number of viable seeds the better. Seed formation is important for plants as it ensures their perpetuation to the next generation, and for humans because a lot of staple food crops we consume are actually seeds or grains of the plant.

The timing of flowering is also important since late flowering can hinder seed-set; too early and the plant will not have sufficient energy to form mature fruits. There are a myriad of endogenous and external factors which control when a plant flowers. The environmental factors include temperature, light conditions and the season. Internal cues like plant age and hormone levels also contribute to it. All these factors converge into creating suitable conditions for the plant to flower (Ausín et al., 2005; Boss et al., 2004; Putterill et al., 2004). The genetic pathways underlying these processes integrate to activate the main activators or florigens. In *Arabidopsis* model species the main activator genes are *FLOWERING LOCUS T (FT)* and *SUPRESSOR OF OVEREXPRESSOR OF CONSTANS 1 (SOC1)* (Kardailsky et al., 1999; Kobayashi et al., 1999; Blasquez and Weigel, 2000; Borner et al., 2000). In all plants studied similar ortholog genes have been found performing similar function.

1.1.1 FLOWERING IN ARABIDOPSIS

Arabidopsis thaliana is considered a good model plant because of its short life cycle, small stature, few number of chromosomes, prolific seed production and comparative ease of mutant generation. It is a facultative long day (LD) plant, which means it flowers fast under long day conditions (16h light, 8h dark) but given enough time it will also flower under short days (SD; 8h light, 16h dark) (Zeevaart, 2009).

There are several genetic pathways involved in flowering including the age, the photoperiod, the gibberellic acid (GA), the vernalization and the autonomous pathways. The crosstalk among all these pathways finely tunes floral induction (Andrés and Coupland, 2012; Song et al., 2013; Mutasa-Göttgens and Hedden, 2009).

The age pathway involves decreasing expression of micro-RNA 156 (miR156) and increasing expression of miR172 allowing juvenile-adult transition to come about (Schwab et al., 2005; Yang et al., 2011; He et al., 2018). Thus, the shoot apical meristem (SAM) gets converted to the inflorescence meristem allowing flowers to develop. miR156, expressed in juvenile leaves, inactivates *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes (Wang et al., 2009). The *SPL* gene family in *Arabidopsis* consists of 16 genes which transcribe transcription factors involved in many plant developmental processes (Cardon et al., 1997; Cardon et al., 1999; Schwarz et al., 2008). Few of them have been shown to play important roles in flowering such as *SPL9* and SPL15 that, when miR156 decreases, promote miR172 expression which in turn inactivates floral repressors such as the *APETALA2-like* genes allowing FT activation (Schwab et al., 2005; Wu and Poething, 2006). *SPLs* also act directly on SOC1 to promote flowering (Wang et al., 2009).

In the photoperiod pathway, CONSTANS (CO) acts as a major flowering inducer in *Arabidopsis* (Samach et al., 2000; Suárez-López et al., 2001). Light perception by phytochrome A (PHYA) in leaves leads to CO expression in the vasculature. CO is a photostable B-box zinc finger protein which peaks and stabilizes at dusk (Putterill et al., 1995; Robson et al., 2001; Khanna et al., 2009). CYCLING DOF FACTORS (CDFs) in conjunction with TOPLESS (TPL) inhibit CO expression (Liu and Karmarkar, 2008; Fornara et al., 2009; Goralogia et al., 2017). A FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) and GIGANTEA (GI) complex targets CDFs for degradation thereby leading to CO upregulation (Fowler et al., 1999; Mizoguchi et al., 2005; Sawa et al., 2007; Imaizumi

et al., 2005). CO then activates *FT* and *TWIN SISTER OF FT (TSF)* (Samach et al., 2000; Yamaguchi et al., 2005). FT and TSF are long range mobile signals, which travel from leaves to the shoot apical meristem (SAM), interact with FD (a bZIP protein) and trigger expression of *SOC1* and *LEAFY (LFY)* (Abe et al., 2005; Lee and Lee, 2010).

The GA pathway promotes flowering by indirectly allowing the activation of *FT*, *SOC1* and also *LFY* (Wilson et al., 1992; Koornneef et al., 1991; Moon et al., 2003; Blázquez et al., 1998). Under SD, the hormone GA plays a prominent role in determining flowering time (Huang et al., 1998; Coles et al., 1999). DELLA proteins prevent activation of *FT*, *TSF* and *SOC1*. GA's promote DELLA degradation thereby inducing flowering (Yu et al., 2012). This occurs both under LD and SD, but in LD the rapid effect of CO masks the role of GAs. GAs also upregulates *LFY* directly through a MYB-like transcription factor (Gocal et al., 2001; Eriksson et al., 2006).

The vernalization and autonomous pathways act by repressing a repressor*, FLOWERING LOCUS C (FLC)*, of the florigens (Lee et al., 1993; Michaels and Amasino,1999; Sheldon et al., 1999). Exposure to long lasting cold conditions (vernalization) induces flowering in many species, including some accessions of *Arabidopsis*. This treatment decreases the levels of *FLC*, in turn upregulating *FT* and *SOC1*. FLC, a MADS-box transcription factor, is upregulated by FRIGIDA (FRI) of the autonomous pathway (Sheldon et al., 2000; Johanson et al., 2000; Geraldo et al., 2009; Zhu et al., 2021). Several genes from the autonomous pathway including *FVE* (an *Arabidopsis* homolog of the retinoblastoma associated protein), *FPA*, *FY*, *FCA*, *FLOWERING LOCUS K* (*FLK*) all contribute to *FLC* repression (Ausin et al., 2004; Kim et al., 2004; Simpson, 2004; MacKnight et al., 1997; Simpson et al., 2003; Lim et al., 2004; Schomburg et al., 2001; Li et al., 2008).

The ambient temperature also regulates flowering time. An increase in ambient temperature leads to early flowering in some *Arabidopsis* accessions. Exposure to higher temperature (27°C) in short day conditions also leads to *Arabidopsis* flowering earlier regardless of the photoperiod. The histone variant H2A.Z plays a role here, where the promoter of *FT* has lower occupancy by H2A.Z, allowing the flowering machinery access to the promoter and induce the process (Kumar and Wigge, 2010; Talbert and Henikoff, 2014). SHORT VEGETATIVE PHASE (SVP), which is a repressor in the flowering time pathway, is also degraded at a higher temperature (Lee et al., 2007; Fernández et al., 2016).

All these and other floral induction regulating pathways are not independent and many cross-talks at different points are found (Figure 1). For example, regulation of *FT* occurs not only by CO in the photoperiod pathway but also by FLC through the vernalization and autonomous pathways. In turn, *FLC* is upregulated by FRI of the vernalization pathway but downregulated by FVE and FLK of the autonomous pathway.

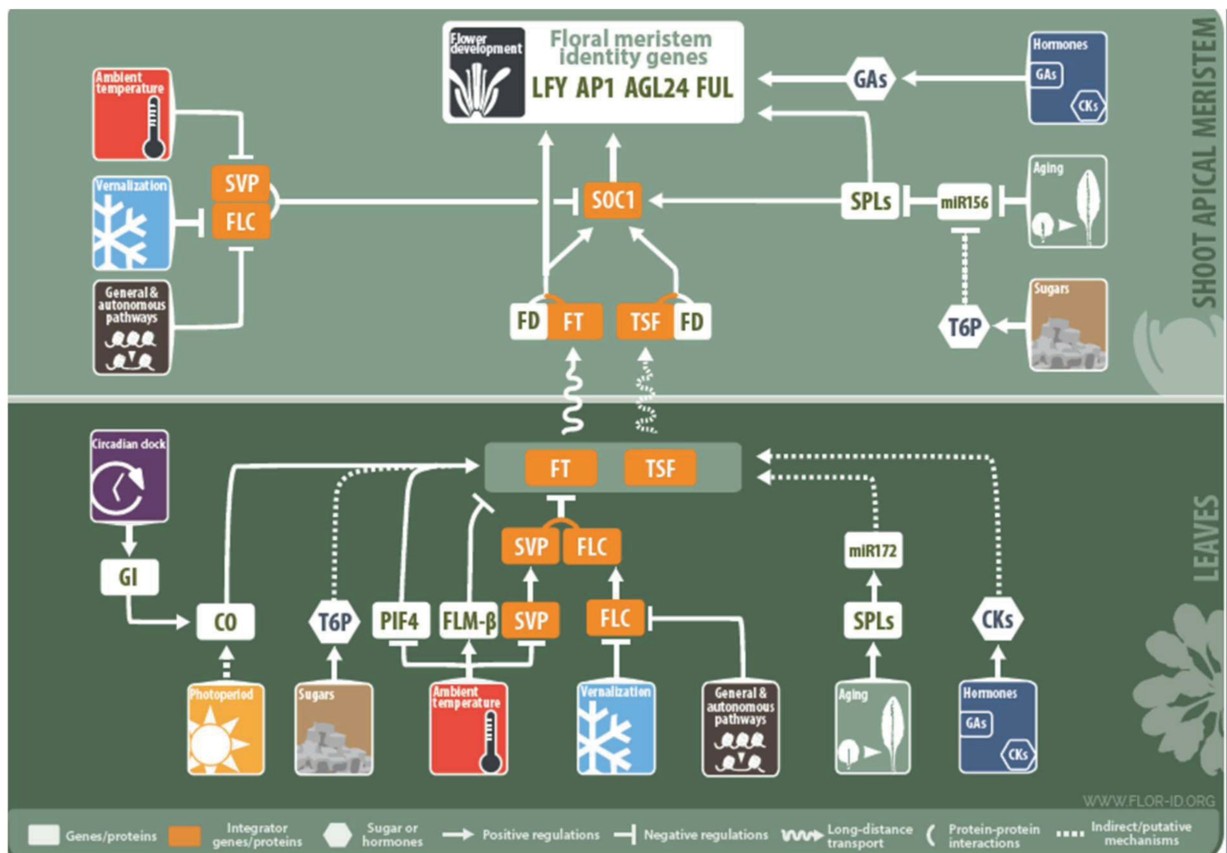


Figure 1: Flowering pathways in *Arabidopsis*

Modified from Flor-ID (Bouché, Lobet et al. 2016)

1.1.2 CO EFFECT IN PROMOTING FLOWERING IN ARABIDOPSIS

The *FT* promoter is a long complex promoter with 4 cis-regulatory regions, namely, the proximal 5’ untranslated region (5’UTR), the CO responsive elements 1&2 (CORE1&2) and the distal CCAAT box and E-box, from the closest to the ATG to the farthest. The COREs consist of CORE1 (-220 bp) (TGTGA), CORE2 (-161 bp) (TGTGG) and 2

palindromic sequences P1 (-267 bp) (CCACA) and P2 (-285 bp) (TGTGG). All 4 elements are recognized directly by CO (Adrian et al., 2010; Tiwari et al., 2010; Cao et al., 2014, Lv et al., 2021).

CO is a protein with 2 domains, an N-terminal B-box domain and a C-terminal CCT domain. It lacks a specific DNA binding domain, which means that it needs help from other proteins to bind to a gene and to perform its function. CO forms a part of protein complexes in order to regulate *FT* (Ben-Naim et al., 2006; Wenkel et al., 2006). The CCT domain of CO is homologous to the conserved domain in the NUCLEAR FACTORY Y (NF-Y) protein. It is through this that an NF-Y/CO complex is formed and binds to the *FT* COREs (Gnesutta et al., 2017). The NF-Y protein is a trimeric complex with subunit NF-YA being DNA binding and NF-YB and NF-YC being histone fold domain (HFD) proteins (Romier et al., 2003; Huber et al., 2012; Nardini et al., 2013). CO binds to the NF-YB/YC elements due to the homology between its CCT domain and NF-YA and now a tetrameric protein complex is formed. The NF-YA then binds to the distal *FT* promoter sequence CCAAT. A loop in the *FT* promoter DNA (through another region or yet unidentified proteins) brings the distal and proximal elements of the promoter closer and the CO-CCT binds to the CORE2. These bindings are enhanced by the NF-YB/NF-YC subunits. The binding of CO to COREs is majorly responsible for upregulation of *FT* and subsequent floral transition (Kumimoto et al., 2010; Cao et al., 2014; Hou et al., 2014; Siriwardana et al., 2016; Swain et al., 2017).

CO expression occurs diurnally corresponding to the photoperiod. Circadian clock genes like *GI*, *LHY* (*LATE ELONGATED HYPOCOTYL*), *FKF1* and *CDFs* also influence flowering. Some mutants with aberrations in the circadian clock genes also show abnormal flowering times. Therefore, there is a mechanism which affects both these pathways with CO being the common link in regulating flowering. *CO* mRNA levels under LD peak at ZT16 and dawn. These levels oscillate within a period of 24 hours showing entrainment by the circadian clock. Under SD, CDFs repress *CO* from being expressed and this repression is maintained because GI accumulates at dusk whereas FKF1 does it after dusk and the functional GI-FKF complex is not formed. Under LD however, *GI* and *FKF1* both peak at the same time when it is light, forming a complex that degrades CDF proteins, leading to the promotion of *CO* transcription. Light signalling through phytochromes and cryptochromes stabilizes the CO protein, allowing it to get to the threshold required for binding to FT and inducing its expression (Figure 2). Therefore, *FT* mRNA levels also

showed a similar pattern to CO active protein (Suárez-López et al., 2001; Valverde et al., 2004; Imaizumi et al., 2005; Sawa et al., 2007).

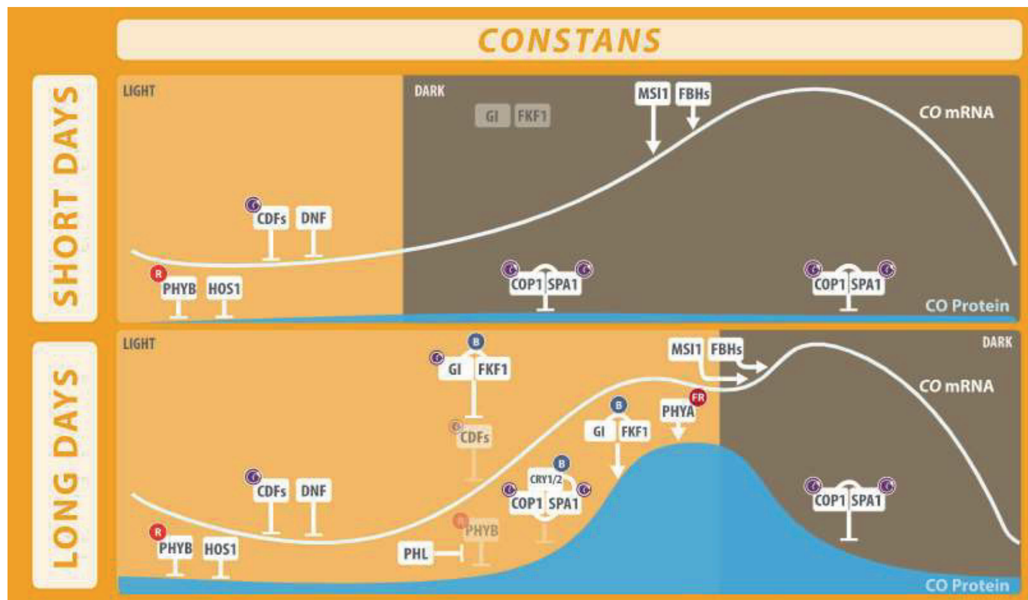


Figure 2: Diurnal oscillation of CO

Figure from Flor-ID (Bouché, Lobet et al. 2016)

1.1.3 FLORAL REPRESSORS

Like in all gene regulatory networks, repressors as well as activators are required to maintain balance. FLC is the major identified and studied floral repressor. It directly suppresses *FT* and *SOC1*, although it does not do so alone. SHORT VEGETATIVE PHASE (SVP), a MADS protein, interacts with FLC and is also involved in maintenance of the vegetative state of the plant (Hartmann et al., 2000; Jang et al., 2009; Li et al., 2008). FLC and SVP functions depend on each other and they act on the *SOC1* promoter and the first intron of *FT* to suppress these genes directly while also indirectly blocking *FD* upregulation (Mateos et al., 2015; Marín-González et al., 2015). Environmental conditions also play a role in regulating flowering. For example, CDFs repress *CO* expression in the morning through *TPL* (Goralogia et al., 2017). In the evening, however, this repression is released by targeted degradation of CDFs by FKF1, ZEITLUPE (ZTL) and LOV KELCH PROTEIN2 (LKP2)

redundantly (Fornara et al., 2009). The AP2-like family of proteins including AP2, three TARGET OF EAT (TOE1, TOE2 and TOE3) proteins, SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (SNZ), whose genes are translationally repressed by miR172, also act as floral repressors (Aukerman and Sakai, 2003; Schmid et al., 2003). Two proteins TEMPRANILLO 1 (TEM1) and TEM2, belonging to the RAV (Related to ABI3/VP1) family of transcription factors (TFs) prevent precocious flowering by transcriptional repression of *FT* (Castillejo and Pelaz, 2008; Lee et al., 2007). Both *TEM1* and *TEM2* are directly up-regulated by SVP, however at low ambient temperatures SVP only regulates *TEM2* specifically (Marín-González et al., 2015). Recently, another family of TFs, MYCs, have also been shown to play a role in floral repression (Zhai et al., 2015; Wang et al., 2017; Bao et al., 2019; Wang et al., 2020).

1.1.4 MYCs ROLE IN FLOWERING

MYC2, MYC3 and MYC4 belong to the basic helix loop helix (bHLH) family of transcription factors and are involved in many pathways but are key in jasmonic acid (JA) mediated biotic stress responses (Boter et al., 2004; Lorenzo et al., 2004). They have a bHLH domain at the C-terminal and a putative transcriptional activation domain (TAD) at the N-terminal. These TFs have a role in hormone signalling pathways, especially the JA pathway. During normal conditions, JASMONATE-ZIM DOMAIN (JAZ) repressors inhibit MYC2, MYC3 and MYC4 function (Chini et al., 2009). When faced with biotic stress, the production of JA-Ile releases this MYC repression, which then act in downstream pathways to activate defence responses (Fernández-Calvo et al., 2011; Chen et al., 2012). This mechanism is also involved in delaying flowering. It has been proven that MYC2/3/4 are required for JA mediated delayed flowering response, as *myc2/3/4* exhibited an early flowering phenotype which was maintained even on application of exogenous JA (Wang et al., 2017). Wang et al. (2017) also showed that MYC2 represses *FT* expression by direct binding. Under SD, MYC3 has been shown to supress flowering by binding directly to *FT* (Bao et al., 2019). Therefore, MYCs also act as floral repressors (Zhai et al., 2015; Wang et al., 2017; Bao et al., 2019; Wang et al., 2020).

1.2 RAV GENES

There are 6 *RAV* genes in *Arabidopsis*: *RAV1*, *RAV1-like*, *RAV2*, *RAV2-like*, *RAV3* and *RAV3-like* (Riechmann et al., 2000). *RAV2-like* and *RAV2* have been renamed *TEM1* and *TEM2* respectively because their loss of function mutants (*tem1-1* and *tem2-2*) caused an early flowering phenotype (tempranillo = early in Spanish) (Castillejo and Pelaz, 2008). RAV transcription factors are characterized by having 2 main domains: a C-terminal B3 domain that recognizes CACCTG and an N-terminal AP2/ERF domain that recognizes CAACA (Kagaya et al., 1999; Yamasaki et al., 2004; Waltner et al., 2005; Riechmann and Meyerowitz, 1998). They also share another domain which contributes to their repressive activity called the B3 repression domain (BRD), the core of this is a consensus sequence of 5 amino acids R/KLFGV (Ikeda and Ohme-Takagi, 2009).

RAV genes of different species are involved in a myriad of aspects of plant development. Role of *RAV* genes in growth inhibition has been studied in many species including *Arabidopsis* where *TEM1* and *TEM2* over-expressor lines cause dwarfism (Castillejo and Pelaz, 2008). RAV1 might also play a role in promoting leaf senescence and indirectly regulating seed germination in *Arabidopsis* (Hu et al., 2004; Woo et al., 2010). TEM1 and TEM2 have been proven to play a role in salt tolerance in *Arabidopsis* (Osnato et al., 2021). TEM homologs in poplar hybrids have also been shown to have a role in growth cessation, preventing premature bud burst and bud set (Moreno-Cortés et al, 2012). In rice, OsRAV9/OsTEM1 plays a similar function to AtTEM1/2 of floral repression while OsRAV11/12 have roles in gynoecium development (Osnato et al., 2020).

1.2.1 TEM GENES IN FLOWERING

TEM protein levels differ throughout the life cycle of a plant. In seedlings, there is a high amount of *TEM* mRNA level which declines dramatically at floral transition at the time that *FT* mRNA levels increase and leads to flowering. This happens at days 10-12 in wild-type plant whereas in *tem1-1* and *tem2-2* mutants, it happens much earlier at around 6 days. This shows an inverse relationship between TEM and FT proteins (Castillejo and Pelaz, 2008). TEM protein accumulation follows the diurnal pattern of *TEM* mRNA (Osnato et al., 2012).

RAV binding sites in the 5'UTR (untranslated region) of the *FT* promoter hinted at the *FT* repression by TEMs being mediated by direct DNA binding. TEM binding to *FT* was confirmed by gel-shift and chromatin immunoprecipitation (ChIP) assays. Interestingly, the TEM binding site is very close to the CO binding site. This could mean that in LD, CO (activator) and TEM (repressor) compete to bind to the *FT* promoter and *FT* levels are a result of their quantitative balance (Kagaya et al., 1999; Wenkel et al., 2006; Castillejo and Pelaz, 2008).

Regardless of CO being inactive under SD, *tem* mutants flower earlier in non-inductive conditions as well. This suggested a role of TEMs in the GA pathway. As mentioned, GA is the major inductor of *SOC1* and thereby of flowering under SD. In TEM over-expressors 35S:*TEM1* and 35S:*TEM2*, significant reduction in *GA3OX1* and *GA3OX2* was observed, whereas in *tem1-1* and double mutant *tem1-1tem2-2* upregulation of *GA3OX1* and *GA3OX2* was found. ChIP experiments also showed TEM1 binding to a RAV binding site in the first exon of *GA3OX1* and *GA3OX2*. This means that TEMs directly repress GA biosynthesis and delay flowering (Castillejo and Pelaz, 2008; Osnato et al., 2012).

1.2.2 REGULATION OF TEM GENES

miR172 in the age dependent pathway represses targets TFs like APETALA2 (AP2) and SCHLAFMUTZE (SMZ) (Zhu and Helliwell, 2011). These TFs also bind *TEM1* DNA in ChIP-ChIP experiments. This means that the targets of the age dependent pathway have a role in controlling *TEM* expression (Mathieu et al., 2009) and at the same time TEMs repress *MIR172* genes (Aguilar-Jaramillo et al., 2019).

TPL and TPL-related (TPR) proteins are a family of widespread transcription factors which act as transcriptional-corepressors in a number of processes in *Arabidopsis*. Interestingly, all RAV proteins except RAV1L have also been demonstrated as TPL/TPR interactors further supporting the repression function of TEMs, likely because of the interaction with these co-repressors (Long et al., 2006; Szemenyei et al., 2008; Causier et al., 2012).

EARLY FLOWERING 3 (ELF3) is a repressor of flowering at low temperatures. *elf3* mutants have lower levels of *TEM2* at both ambient and low temperatures suggesting that ELF3 acts in increasing *TEM2* expression for its response (Strasser et al., 2009).

SENSITIVITY TO RED LIGT REDUCED1 (SRR1), a protein involved in circadian clocks (Staiger et al., 2003), also promotes *TEM1* and *TEM2* expression. *srr1-1* mutant plants have an early flowering phenotype, higher *FT* and lower *CDF* levels (Johansson and Staiger, 2014).

Some transcription factors like FLC and SVP bind directly to regulatory regions of *TEM1*, indicating they might directly affect *TEM* expression (Deng et al., 2011; Tao et al., 2012). FLC and SVP both supress flowering. SVP has been shown to upregulate *TEM1* and *TEM2* expression through DNA binding (Tao et al., 2012, Marín-González et al., 2015). SVP also directly regulates *TEM2* under low ambient temperatures (Marín-González et al., 2015) SOC1, a flowering promoter, probably does the opposite just to stop the floral repression activity of TEMs to allow progression of floral development as they are downregulated by SOC1 (Tao et al., 2012).

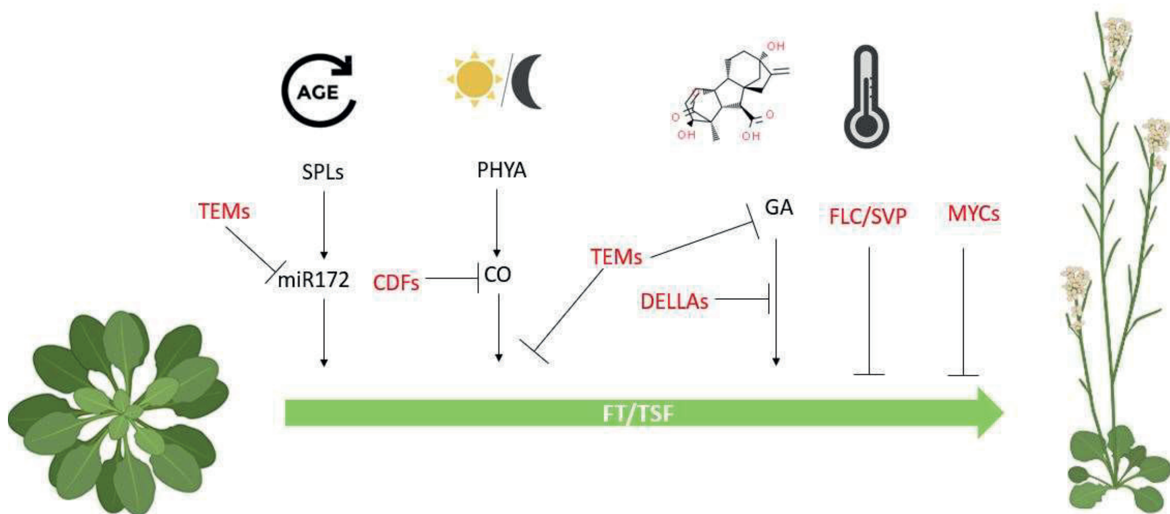


Figure 3: Key genes in the regulation of *FT* and *TSF* of *Arabidopsis thaliana*

1.2.3 RAV GENES IN EVOLUTION

RAV genes, despite playing a key role in flowering, are found in non-flowering plants as well, including the moss *Physcomitrium patens*. To better understand the evolution of this gene family, our lab studied their presence throughout the tree of life and interestingly found

that they are present in all species of land plants. RAV roles in a diverse array of developmental processes as well as in stress response across multiple species may also indicate TEM involvement in some of these aspects. *TEM* homolog genes of numerous flowering plants have been identified as floral repressors but they have also been found to play a major role in other processes, which may account for the presence of these genes before the evolution of flowering. And although angiosperms have the presence of a flower as a prevalent feature, they are otherwise quite distinct species. Despite that, the repressive role of TEMs seem to be shared among them, though to what extent still remains to be studied.

TEM homolog genes have already been identified in both gymnosperms like *Pinus* and angiosperm trees including chestnut (*Castanea sativa*) and poplar (*Populus trichocarpa*). However, no information on their roles in flowering of trees has been published.

1.2.4 RAV ROLES IN DIFFERENT PLANT FAMILIES

RAV genes in divergent plant species have a multitude of roles in plant development not limited to their role in flowering. *GmRAV* (*Glycine max*) expressed in tobacco causes an overall decrease in plant growth (Zhao et al., 2008). Soybean with 13 *RAV* genes divided into 3 phylogenetic classes has varied expression patterns in drought and salt stresses (Zhao et al., 2017). GmRAVs are also related to photosynthesis (Zhao et al., 2008), cytokinin signalling and photoperiod regulation (Zhao et al., 2012). Soybean RAV1s have also been shown to regulate root regeneration (Zhang et al., 2018).

In tomato, SlRAV2's role in increased resistance to bacterial wilt has been revealed (Li et al., 2011). The pepper CaRAV1 induction by pathogen infection and osmotic stress leading to increased resistance implicates it as one of the factors responsible in stress mediated responses (Lee and Hwang, 2006; Sohn et al., 2006). RAVs in cassava, MeRAV1 and MeRAV2, also contribute to resistance against bacterial blight (Wei et al., 2017). 15 Rice RAVs from 4 phylogenetic classes are involved in various signalling pathways and during virus infection (Chen et al., 2021). Cotton, melon and tobacco *RAV* genes also seem to confer resistance during salt stress (Li et al., 2015; Zhao et al., 2019; Gao et al., 2020). Out of the 11 identified RAVs in pear, two of them PbRAV6 and PbRAV7 seem to play a role in abiotic

stress responses, and PbRAV6 is involved in anthocyanin development and helps regulate fruit pericarp colour (Liu et al., 2021). In strawberries, anthocyanin biosynthesis has been indirectly promoted by FaRAV1 (Jin et al., 2017).

All these varied functions of *RAV* genes seem to suggest that throughout the course of evolution, this family gained different abilities to bind to and affect gene expression in different species and different physiological processes.

1.2.5 EVOLUTION OF RAV GENE STRUCTURE

Gene families along plant lineages evolve in several ways including duplication and transposition of DNA segments (Moore and Purugganan, 2003; Cannon et al., 2004). In pear, 2 pairs of tandem repeats and 3 pairs of segmental duplication have been discovered suggesting that in genus *Pyrus*, these events played a major role in *RAV* family evolution (Liu et al., 2021).

The presence or absence of introns might also contribute to this. *RAV* genes in *Arabidopsis* do not have introns whereas in rice and cotton, around 20% of *RAV* genes have been found to have them (Chen et al., 2021; Kabir et al., 2022). Generally, gene families with no introns are considered conserved and the presence of introns hints at evolution and gain of new functions (Roy and Gilbert, 2005; William Roy and Gilbert, 2006).

1.2.6 POPLAR RAVs

Roles of genes homologous to an annual gene in perennials are slightly more divergent because unlike their annual counterparts, perennial life cycles years through different climatic conditions. RAVs are no different. *RAV* paralog genes in poplar (*CsRAV1*), a perennial, might take part in repressing bud outgrowth by causing early sylleptic branching in poplar hybrids (Moreno-Cortés et al., 2012). FTs in *Populus* are involved in controlling growth as well as growth cessation and bud dormancy (Böhlenius et al., 2006; Hsu et al., 2006; Ruttink et al., 2007). Since *TEM* genes are a key player in the CO/FT module in *Arabidopsis*, it stands to reason that *RAV* genes in poplar could also have a similar function and be involved in growth cessation. In poplar there are 2 paralogs closest to *Arabidopsis*

*TEM*s i.e., *PtRAV1* and *PtRAV2*, both containing the B3 and AP2 domains. Their expression patterns also correlated with *Arabidopsis TEMs* being expressed ubiquitously. *PttRAV1&2* RNAi lines showed a higher amount of sylleptic branching (Thesis- Esther Marín-González). *In silico* promoter analyses of *PtFT1* and *PtFT2* also showed these genes to be possible *PtRAV* targets (Thesis- Esther Marín-González). With these similarities between *Arabidopsis* TEMs and poplar RAVs, we thought it would be interesting to observe if the latter's putative role in flowering was conserved.

1.3 TRANSCRIPTION FACTORS AND THEIR MODE OF ACTION

Transcription factors are relatively small proteins that bind directly to DNA and control spatio-temporal gene expression. These proteins might act alone or in complexes to induce or supress transcription. They are involved in all regulatory processes from basic physiological growth to stress responses. Some TFs are ubiquitous and present in all cells of an organism, and some are cell or tissue specific. TFs have a DNA binding domain and the ones with a consensus sequence of a particular domain form a family. There are numerous TF families, some present universally and some are taxa specific. A family usually has conserved functions and mode of actions. (Latchman, 1997)

TFs can affect gene expression in a variety of ways. They can act as activators and recruit other TFs, an enzyme or themselves kick start transcription of a gene. They can act as repressors and recruit other TFs, block binding of an enzyme or another TF or alone stop the expression of a gene. They can also modify epigenetic marks on histone proteins or on the chromatin. In some cases, TFs act as enhancers and increase rate of transcription and in others as silencers and decrease the rate, by binding to specific sequences of a gene (Latchman, 1997; Babu et al., 2004).

1.3.1 CO, TEM and MYC BINDING

TEM1 and TEM2 act as floral repressors in multiple flowering regulating pathways (Castillejo and Pelaz, 2008; Osnato et al., 2012; Marín-González et al, 2015; Aguilar-Jaramillo et al., 2019). Since *Arabidopsis* is a facultative LD plant, flowering occurs much

faster in these favourable conditions, largely due to the role of CO. CO induces *FT* expression which triggers the flowering initiation pathway in *Arabidopsis*. This induction happens due to direct binding of CO protein complex to regulatory regions in the *FT* promoter (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et a., 2000). TEM repression also occurs due to direct binding to the 5'UTR region of the *FT* promoter, to RAV binding sequences CAACA and CACCTG are at -43 bp of the *FT* promoter (Castillejo and Pelaz, 2008). The regulatory region where TEM binds is very close to the region where CO binds to the *FT* promoter (CORE 1&2) (Adrian et al., 2010; Osnato et al., 2012).

TEM and *MYC* expression also show a diurnal oscillation like CO, with low levels during the day and a peak around ZT16 (Castillejo and Pelaz, 2008; Bao et al., 2019). So, there is a quantitative balance between CO, an upregulator, and TEMs, downregulators, which controls the timing of flowering under LD.

MYCs are major TFs involved in the JA mediated defence responses (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Qi et al., 2011). However, *myc2/3, myc2/4* and *myc2/3/4* mutants have been shown to have an early flowering phenotype. *myc3/4* and the single mutants on the other hand do not have a significant effect on flowering, indicating that the three genes act somewhat redundantly and that MYC2 plays a slightly stronger role in this mechanism. The early flowering phenotype of the mutants is due to having higher levels of *FT* and *TSF* (florigens) genes (Wang et al., 2017). The expression patterns of *MYC* and *FT* are also similar. MYCs act by binding to G-box or G-box like elements of which the *FT* gene has plenty. MYC2 has been proven to bind to two of these elements in the *FT* gene, one around the *FT* transcription start site (TSS) region and the other further downstream. So, MYC TFs might be direct repressors in the flowering pathway by binding to the regulatory regions of *FT* (Wang et al., 2017). Bao et al. (2019) showed that under SD, MYC3 competes with CO to bind *FT* to control flowering time, and that this repression is maintained by DELLAs and released by GAs (Bao et al., 2019).

1.3.2 GENE REGULATION BY HISTONE MODIFICATIONS

Eukaryotic gene expression is tightly controlled and spatio-temporally dependent. This happens by either gene activation or gene repression, facilitated by TFs that act in different ways. Other ways in which gene expression is regulated is through post-translational histone

modifications. Histone modifications are temporary changes to some amino acids, usually at the N-terminal tails, that can either activate or silence transcription of a gene (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Berger, 2007; Kouzarides 2007; Lee et al, 2010). These modifications include methylation, acetylation and ubiquitination (Swygert and Peterson, 2014; Bannister and Kouzarides, 2011).

Histone acetylation is regulated by two kinds of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs), performing two opposite functions of adding and removing acetyl groups respectively (Berger, 2007). Histone acetylation is generally considered an active chromatin mark and plays a role in controlling flowering time in *Arabidopsis*. For example, *FLC*, of the vernalization and autonomous pathways, is deacetylated by FVE acting as a *FLC* repressor; in *fve* mutants *FLC* is more acetylated, therefore, it is more transcriptionally active which leads to a late flowering phenotype (Ausín et al., 2004). Histone deacetylation also occurs by *FLOWERING LOCUS D* (*FLD*), a gene in the autonomous pathway (He et al., 2003). HDA6 affects flowering time by interacting with FLD (Yu et al., 2011).

Trimethylation of histone H3 lysine 27 (H3K27me3) is a very important epigenetic mark. It was first discovered in Drosophila and is deposited by the Polycomb Repressive Complex 2 (PRC2) (Schwartz et al., 2006; Bowman et al., 2014). The PRC1 and PRC2 are complexes of the Polycomb group proteins (PcG), first studied in *Drosophila* (Lewis, 1949). They are mainly involved in gene silencing (McKeon and Brock, 1991).

In plants, around 5% of the canonical histone H3.1 contains H3K27me3 and *Arabidopsis* mutants defective in this, have serious developmental problems (Johnson et al., 2004; Ahmad and Henikoff, 2002; Schubert et al., 2005; Lindroth et al., 2004; Kinoshita et al., 2001). H3K27me3 is considered a repressive mark and is associated with silent genes generally in euchromatic regions (Zhang et al., 2007; Roudier et al., 2009; Cao et al., 2002; Liu et al., 2010). Histone methylation is carried out by the SDG (SET domain group) histone methyl transferases that contain the 130 aa SET domain.

In *Arabidopsis*, H3K4 and H3K36 regions of the active *FLC* are highly methylated, whereas H3K9 and H3K27 methylation increases in repressed *FLC* (Sung and Amasino, 2004).

CURLY LEAF (CLF), an SDG protein that forms part of the PRC2, directly repressed *FLC* and *FT* by H3K27me3 (Jiang et al., 2008).

1.3.3 TEM AND MYC POSSIBLE RELATIONSHIP

TEMs and MYCs are both proven floral repressors and bind directly to regions around the 5'UTR of the *FT* promoter (Castillejo and Pelaz, 2008; Wang et al., 2017). But how exactly this repression occurs is not known. MYC2 is an established key regulator in the JA response pathway (Zhao et al., 2013; Aleman et al., 2016; Chen et al., 2011; Kazan and Manners, 2013). HDA6 has also been implicated to play an important role in the same JA pathway. *axe1-5* (*Arabidopsis* HDA6 mutant) and HDA6-RNAi lines show a late flowering phenotype, possibly due to *FLC* upregulation. The *FLC* levels in these late flowering mutants were higher than in the WT and it also showed histone H3 hyperacetylation (Wu et al., 2008). Therefore, HDA6 and MYCs both play a role in the JA and flowering pathways.

MYC2 physically interacts with jasmonate ZIM domain (JAZ) repressor proteins in its role as an activator in the JA signalling pathway (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Chen et al., 2011; Fernández-Calvo et al., 2011; Kazan and Manners, 2013; Schweizer et al., 2013). *Arabidopsis* JAZ proteins have been proven to interact with TPL and TPRs to act as co-repressors in the JA pathway (Pauwels et al., 2010). TPL and four TPRs belong to Groucho/Tup1 group of corepressor proteins which are recruited directly or indirectly by multiple other transcription factors in different pathways (like JA mediated response pathway) to control transcription (Kieffer et al., 2006; Long et al., 2006). The probable mechanism of repression of the TPL/TPR family is to act in concert with HDACs to produce a repressed chromatin state (Long et al., 2006). TPL/TPR interactome data shows their direct binding to SOC1 as well as RAV family members TEM1, TEM2 and RAV1 (Causier et al., 2012). All these factors contribute in making HDA6 and TPL common players in both flowering and JA pathways involving TEM and MYC.

1.4 EPIGENETIC REGULATION OF FT

As central in inducing flowering, the epigenetic regulation of *FT* deserves a specific section. In *Arabidopsis*, CLF, a PRC2 methyltransferase component that deposits H3K27me3 marks, acts on *FT*, placing a gene silencing mark on it (Goodrich *et al.*, 1997; Jiang *et al.*, 2008; Lopez-Vernaza *et al.*, 2012). This methylation mediated silencing is maintained by LIKE HETEROCHROMATIN PROTEIN1 (LHP1), which binds to methylated sites through its chromodomain (Goodrich *et al.*, 1997; Turck *et al.,* 2007; Zhang *et al.,* 2007; Exner *et al.,* 2009; Adrian *et al.,* 2010). The formation of the CO complex with NF-YA/YB/YC and its subsequent binding to *FT* disturbs the action of LHP1 and allows activation of FT (Liu et al., 2018; Luo et al., 2018). Jumonji-class TF, RELATIVE OF EARLY FLOWERING 6 (REF6), an H3K27 demethylase, also helps in activating *FT*. It does so indirectly by repressing *FLC* (Noh et al., 2004; Lu et al., 2011).

H2DC histone deacetylase also targets *FT* towards the end of the day to repress its transcription and prevent overexpression of *FT*. It does so in an MRG1/2 (methylation readers) dependent manner. Moreover, H2D2C and CO compete to bind to MRG1/2 and affect *FT* expression (Guo et al., 2020; Bu et al., 2014).

The ATPase dependent chromatin remodeller PICKLE (PKL) interacts with CO and aids its binding to the *FT* promoter. This action of PKL at dusk leads *FT* transcription and thus, induces flowering (Ogas et al., 1999; Jing et al., 2019).

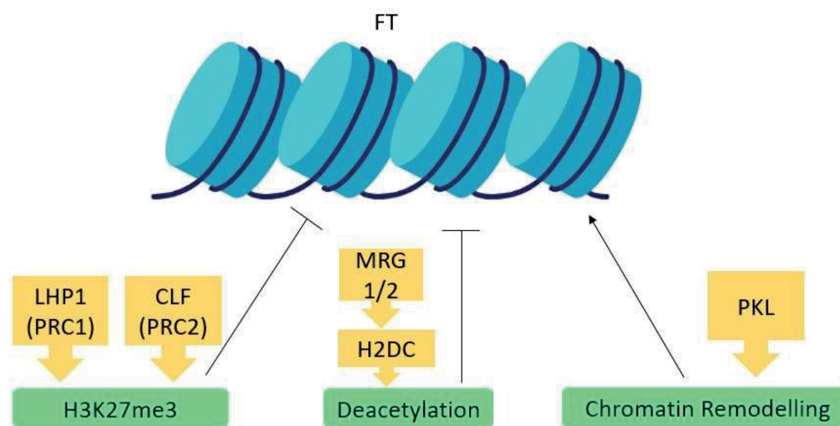


Figure 4: Key Epigenetic Regulators of *FT*

1.4.1 FT LOOPING

In *Arabidopsis*, CO binding to *FT* requires the NF-Y family of transcription factors. These factors recognize the CCAAT box in CO and facilitate binding. The NF-YA subunit directly binds to the DNA by inserting an alpha helix in the minor groove whereas the NF-YB/YC form heterodimers through their histone fold domains and bind to the DNA sugar-phosphate backbone and either the NF-YA or CO proteins. The *FT* promoter contains 2 regulatory regions relevant to this process, a distal CCAAT box and proximal CORE 1&2. It is hypothesized that CO competes with NF-YA to bind the NF-YB/NF-YC dimer. The NF-Y trimeric complex binds to the CCAAT box region of the *FT* promoter and the CO-NF-YB/YC complex binds to the CCACA through the NF-Y proteins. A DNA loop is formed to bring the CCAAT box complex close to the CORE region and bridge the ~5kb distance. The exact mechanism and proteins that facilitate this are not yet known however an intermediary DNA complex with both regions has been found, giving weight to this theory. The CO then binds to the CORE region and promotes *FT* transcription (Gusmaroli et al., 2002; Wenkel et al, 2006; Ben-Naim et al., 2006; Cai et al., 2007; Kumimoto et al., 2008; Kumimoto et al., 2010; Tiwari et al., 2010; Adrian et al., 2010; Cao et al., 2014; Liu et al., 2014; Siriwardana et al., 2016; Gnesutta el al., 2017a; Gnesutta et al., 2017b) (Figure 5).

The A. thaliana has ~10 genes encoding each NF-Y subunit (Petroni et al., 2012), but the NF-Y proteins have also been found to interact with CO homologues in rice and wheat (Li et al., 2011; Kim et al., 2016; Hwang et al., 2016). In rice, a short-day plant, the NF-Y proteins perform a similar function in regulating flowering with respect to photoperiod where they inhibit flowering under long days (Kim et al., 2016; Hwang et al., 2016).

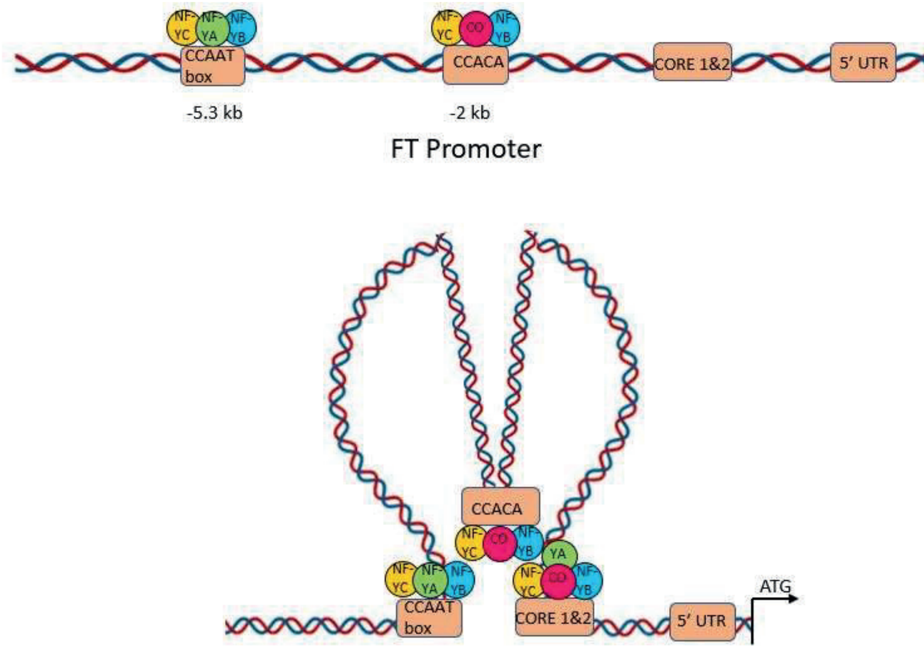


Figure 5: ***FT*** **looping: CO cannot bind and activate** ***FT*** **unless NF-Y proteins help to bring CO to the CORE sites through a** ***FT*** **promoter loop.**

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Arabidopsis thaliana (Col-0, *tem1-1tem2-2*, *myc2myc3myc4* and *tem1-1tem2-2myc2myc3myc4*) were grown on soil/MS+vitamins plates in growth chambers under long-day photoperiod conditions (LD 16h light, 8h dark) and short-day photoperiod conditions (SD 8h light, 16h dark). The soil mixture used was 2:1:1 black peat:perlite:vermiculite and light intensities in the chamber were 230µmol/m^2/s. Seeds were spread on soil/media, stratified at 4°C for 2 days and then transferred to the growth chamber at 22°C.

2.2 Cloning/Construct Formation

pTEM1::HA::TEM1 in pAlligator2– A 1397 bp putative *TEM1* promoter fragment was amplified from *Arabidopsis* genomic DNA using primers 5’-GGGTCGACGCCACGAAGAACTAAATCTGACCG-3’ and 5’-CCGATATCCTCGAGTCTAGAATTTGTTGTGTTTGTGAGAGAG-3’ (with restriction sites for EcoRV, XhoI and XbaI) (Promega, Roche, Roche) and cloned into pGEM-T easy plasmid. This construct was named pIC20 (Promega Biotech Iberia S.L.). The 3xHA tag was amplified from pAlligator 2 (plant transformation destination plasmid) using primers 5’-CCTCTAGACACGCTGACAAGCTGACTC-3’ (with restriction site for XbaI) and 5’-CCCTCGAGTGCATAGTCCGGGACGTCATAG-3’ (XhoI), also cloned into pGEM-T easy and the construct labelled pIC7. Restriction digestion using restriction enzymes HindIII (Roche) and EcoRV was used to excise 2x35S promoter and the 3xHA tag from the pAlligator2 vector. The same enzymes were then used to excise pTEM1 from pIC20 and ligated in pAlligator2 (resulting in pIC30 plasmid). The fragment containing the 3xHA tag was cut from pIC7 and introduced in pIC30 using restriction-ligation with Xba and XhoI to produce pIC33. An LR reaction (Life Technologies S.A.) was done to introduce the *TEM1* cds from TEM1 in pENTR3-C (pMO113, an existing pENTR3c entry vector in the lab containing *TEM1* cds) to pIC33 resulting in pIC41, which contained TEM1 tagged to HA driven by its own promoter, and expressing GFP in the seeds for easier transgenic selection.

pKNAT1::HA::TEM1, pKNAT1::HA::TEM2, pSUC2::HA::TEM1, pSUC2::HA::TEM2 in pAlligator2 – LR reactions were done using pIC34 (containing KNAT1 promoter::HA) and pIC35 (SUC2 promoter::HA) (these plasmids were constructed using the above strategy, where pKNAT1 was amplified from genomic DNA using 5'-CCAAGCTTTAGAGCCCTAGGATTTGACG-3' and 5'-CCGATATCCTCGAGTCTAGAACCCAGATGAGTAAAGATTTGAG-3'; and pSUC2 using 5'-CCAAGCTTGGATCCCCAAAATCTGGTTTC-3' and 5'-CTCGAGTCTAGAATTTGACAAACCAAGAAAGTAAG-3') with pMO113 to produce *pKNAT1::HA::TEM1, pKNAT1::HA::TEM2, pSUC2::HA::TEM1, pSUC2::HA::TEM2* in pAlligator2. TEM2 was amplified from *Arabidopsis* genomic DNA using 5'-GAATTCGGATCCGGCGGAGAAAGATTC-3' and 5'-GGTACCGGGTCGACCTAGTCAAATTGTCT-3'. Restriction-ligation using EcoRI and KpnI was then used to introduce TEM2 into pIC34 and pIC35.

Poplar TEMs – Poplar *TEM1* (**XM_002315922.3**) and *RAV2* (**XM_002311402.3**) (Poplar TEM homologs) were ordered from Twist Biosciences in pTWIST ENTR vectors. LR reaction was done to introduce these genes into pAlligator2.

2.3 Crossing

pMYC2::GFP::MYC2 (Col-0) (kindly donated by Roberto Solanki – CNB, Madrid) (used as female) was crossed with *myc2myc3myc4* and *tem1tem2* to produce heterozygous lines which were backcrossed with the mutants to introduce the pMYC2::GFP::MYC2 construct in the mutants. The plants were genotyped every generation to check for homozygosity.

2.4 Plasmid Isolation

Plasmid isolations were performed using Macherey-Nagel Nucleospin Plasmid kit. (Cultek S.L.U.) 5 ml cell culture was used to pellet cells which were suspended in 500 µl suspension buffer A1, following which 500 µl lysis buffer A2 was added, mixed gently and incubated at RT for 5 minutes. Then 600 µl neutralization buffer A3 was added, mixed and the samples centrifuged at 11000g for 10 minutes. The supernatant was used to bind plasmid DNA to a

column, which was then washed with 600 µl wash buffer A4 and DNA eluted using 50 µl pre-heated elution buffer AE. The plasmid concentration was checked using Nanodrop and it was sent for sequencing to verify the sequence.

2.5 Bacterial Transformation

1 µl isolated plasmid was used to transform competent *Escherichia coli* Top 10 or *Agrobacterium tumefaciens* GV3101 cells using the heat-shock method to transform microorganisms, wherein the mixture of plasmid and bacteria was subjected to short 1 minute incubation cycles at 42°C and ice, 2-3 times to allow entry of the plasmid into the cell. The transformed cells were then grown in LB/YEB liquid culture (supplemented with specific antibiotics) for 1 hour. The cells were spun down, the pellet suspended in very low volumes and then spread on solid media plates with antibiotics to get multiple candidate colonies.

2.6 *Arabidopsis* Plant Transformation

Agrobacterium tumefaciens GV3101 liquid cultures containing the final destination vectors were used to transform *Arabidopsis* using the floral dip method. Flowers and rosette leaves were dipped in a 200ml solution of Agrobacterium supplemented with sucrose (16g) (Fisher) and Silwet L-77 (100µl). Plants were kept in dark to facilitate optimal uptake and then transferred to the greenhouse. Seeds from these plants were collected to select mutants.

2.7 Transgenic Line Selection

Transgenic seeds were selected under the stereomicroscope using GFP as a marker. T1 (Transgenic generation 1) lines were sowed independently and brought to T2. Fluorescence segregation of those lines was checked and those with a single T-DNA insertion, lines with a 3:1 fluorescent: non-fluorescent ratio were used to go into T3. The selected T2 plants were sowed and the subsequent generation with 100% fluorescent T3 seeds were selected as homozygous lines to proceed with.

2.8 Phenotypic analyses

To determine the flowering phenotype, the number of rosette leaves at the time of flowering and total number of cauline leaves was counted on the main stem. Flowering time was also measured as the number of days from germination when the floral bud was visible. Around 10 independent lines were used for each transgenic, and 8 individual plants for each transgenic or mutant plants were scored.

2.9 Protein Isolation and Western Blotting

- Protein Isolation

Around 1 cm^2 of leaf samples from 2-week old plants were collected, frozen in liquid nitrogen and ground using TissueLyserII (QIAGEN) at 30Hz for 1 min. The samples were suspended in 200 ml **extraction buffer (EB)** and centrifuged at 10000g for 10 minutes at 4°C to pellet unground tissue. 100 ml supernatant was transferred to a new tube and the centrifugation repeated. **Loading dye** (final concentration 1X) was added to 50 ml supernatant from this step, heated at 95°C for 5 minutes and centrifuged at 13000 rpm for 1 minute. This protein sample was now ready to be loaded on the protein gel.

- Protein Separation (SDS-PAGE)

Extracted protein samples were separated using SDS-polyacrylamide/bisacrylamide gel electrophoresis (SDS-PAGE) on the basis of their molecular weights. 15 ml of each sample and a molecular marker was loaded in the wells and the gel run at a constant voltage of 175V in 1X **running buffer**.

- Protein Transfer, Membrane Blocking and Washing

The separated proteins were then transferred to a nitrocellulose membrane (Whatman PROTRAN) using a Trans-Blot® Turbo™ Transfer System (BioRad) for 40 minutes at 25V, 1A. Membranes were then blocked using a **TTBS**-5% skimmed milk solution for one hour at room temperature shaking. Blocking solution was discarded and membranes

were incubated overnight at 4ºC shaking in a TTBS-5% skimmed milk solution containing a 1:5000 diluted concentration of Anti-HA-Peroxidase primary antibody (Roche). Membranes were washed 3-6 times in a TTBS solution 1x for 10 minutes shaking.

- Protein Detection and Imaging

Protein detection was done using chemiluminescence. A luminol treatment (Amersham ECLTM Prime Western Blotting Detection Reagent) was applied to membranes and incubated for 5 minutes and revealed using the ImageQuant 800 (Amersham Bioscience). Loading control was checked using 0.5% Ponceau-1% acetic acid solution.

Protein Extraction Buffer (5 ml)

- 100 ml 1M Tris-HCl pH 8
- 150 ml 5M NaCl
- 10 ml 0.5M EDTA pH 8
- 50 ml Triton X-100
- 50 ml 10% SDS
- ½ Complete Protease Inhibitor Tablet (Merck Life Sciences S.L.U.)

5X Loading Dye (10 ml)

- 2.5 ml 1M Tris-HCl pH 6.8
- 5 ml glycerol
- 1g SDS
- 0.76g DDT
- Bromophenol blue added until the solution is blue (0.5g)

Separation Gel (lower) (12%)

- 4 ml Acrylamide/Bisacrylamide (29:1) (Amresco)
- 6 ml Milli Q water
- 3.33 ml lower phase buffer pH 8.8
- 5.34 ml TEMED (Amersham Biosciences)
- 134 ml 10% APS

Stacking Gel (upper)

- 667 ml Acrylamide/Bisacrylamide

- 4334 ml MQ water
- 1667 ml upper phase buffer pH 6.6
- 6.67 ml TEMED
- 67 ml 10% APS

Running Buffer (10X)

- 0.25M Tris
- 1.92M Glycine
- 1% SDS

Transfer Buffer

- 48 mM Tris
- 39 mM Glycine
- 20% Methanol
- 10% SDS

TTBS (1X)

- 25 mM Tris-HCl pH 7.5
- 125 mM NaCl
- 0.2% Tween-20

2.10 Chromatin Immunoprecipitation (ChIP)

50-70 7-day old seedlings were harvested at ZT 16 (peak of TEM expression) under LD and 10-day old seedlings or at ZT 8 under SD, and DNA-protein complexes fixed by vacuum infiltration for 10 minutes at room temperature in 50 ml of **Crosslinking buffer** in 50 ml falcon tubes. Crosslinking was stopped by adding 2.5 ml 2M Glycine (Merck Life Sciences S.L.U.) to every falcon (to a final concentration of 100 mM) and vacuum infiltration continued for 5 minutes. Plant material was rinsed 3 times with sterile water for 10 minutes under agitation at 4°C (by using falcon tubes). Plant material was dried on sterile filter paper, and freeze fixed in liquid nitrogen.

Plant material was then ground to a fine powder and each sample was suspended in 1 ml **Lysis Buffer** and incubated on ice for 10 minutes. Shearing of chromatin was done by sonication (at low power, pulses 30” on, 30” off for 8 times at 4°C) to a length between 300

and 1500 bp. The sample was centrifuged at maximum speed for 10 minutes at 4°C and supernatant transferred to a clean Eppendorf tube. The sonicated chromatin was divided equally into 4/5 tubes, depending on the experiment:

1. INPUT: 200 ml sonicated chromatin to be stored at -80C until de-crosslinking
2. -Ab: 200 ml sonicated chromatin to be used as negative control
3. +Ab: 200 ml sonicated chromatin to be immuno-precipitated with 5 ml of antibody against H3 (Abcam) as positive control
4. +Ab: 200 ml sonicated chromatin to be immuno-precipitated with 5 ml of H3Ac antibody (Millipore) or anti-HA/anti-GFP antibodies (Abcam)
5. +Ab: 200 ml sonicated chromatin to be immuno-precipitated with 5 ml of H3K27me3 antibody (Merck Life Sciences S.L.U.)

The tubes were incubated overnight at 4°C with gentle rotation.

The next day 60 μl Salmon Sperm DNA/Protein A agarose slurry (Merck Life Science S.L.U.) was added to each tube, and incubate for 2 hours at 4°C with gentle rotation to collect the Ab-histone complex. The slurry was pelleted by gentle centrifugation at 4000-5000 rpm for 5 minutes at 4°C, and the supernatant containing unbound, non-specific DNA, was carefully removed. The slurry was washed 6 times (for 3-5 minutes at 4°C with gentle rotation) as follows:

twice with 1 ml **Lysis Buffer**

once with 1 ml **LNDET Buffer**

three times with 1 ml **TE Buffer**

300 μl of freshly prepared **Elution Buffer** (100 mM $NaHCO_3$, 1% SDS) (Sigma) was added to all the tubes, including the stored INPUT samples, and incubated for 30 minutes at room temperature with shaking to release immuno-complexes. 100 μl **Elution Buffer** was added to 200 μl of the INPUT sample, and de-crosslinked in parallel with the immuno-precipitated samples. The samples were centrifuged at maximum speed for 1 minute, supernatant transferred to clean tube, and 12 ml 5 M NaCl (to a final concentration of 200 mM) added. Samples were incubated overnight at 65°C under agitation for reverse crosslinking.

6 ml 0.5 M EDTA, 12 ml 1 M Tris-HCl pH6.5 and 2 ml 10mg/ml Proteinase K (Merck Life Science S.L.U.) was added to each tube and incubated for 1 hour at 42°C. Chromatin was

extracted using equal volume of Phenol:Chloroform:Isoamyl Alcohol (PCI) (25:24:1) (Pancreac Applichem, Merck) by mixing the phases and centrifugation at maximum speed for 5 minutes. 300 µl supernatant was transferred to a clean tube and 700 ml absolute ethanol, 30 ml 3 M NaOAc pH5.2 and 1 ml 20mg/ml glycogen (Thermo Scientific) added. Samples were stored at -20°C overnight.

DNA was pelleted by centrifugation at maximum speed for 10 minutes, and washed with 300 µl 70% ethanol. The supernatant was discarded, the pellet dried and eluted in 100 µl sterile water. This was then used for qPCR analyses.

Crosslinking buffer

- 0.4 M sucrose
- 10 mM Tris-HCl pH 8
- 1 mM EDTA
- 1% Formaldehyde (Merck)
- Protease inhibitors- 1 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml aprotinin

Lysis buffer

- 50 mM HEPES pH 7.5 (Sigma-Aldrich)
- 150 mM NaCl
- 1 mM EDTS
- 1% Triton X-100
- 0.1% SDS
- 0.1% Sodium deoxycholate (Merck Life Science S.L.U.)
- 10 mM Sodium butyrate (Sigma-Aldrich)
- Protease inhibitors- 1 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml aprotinin

LNDET buffer

- 0.25 M LiCl
- 1% NP-40
- 1 mM EDTA
- 1% Sodium deoxycholate

Elution Buffer

- 100 mM $NaHCO_3$
- 1% SDS

2.11 Formaldehyde Associated Isolation of Regulatory Elements (FAIRE)

FAIRE assay was performed as described in Omidbakhsfard et al. 2014, as follows.

Two sets of 50-70 7-day old seedlings were harvested at ZT 16 (peak of TEM expression) under LD or 10-day old seedlings at ZT 8 under SD. DNA-protein complexes were fixed by vacuum infiltration for 10 minutes at room temperature in 50 ml of **Buffer 1 (supplemented with 1% formaldehyde)** in 50 ml falcon tubes in set 1 (FAIRE sample) and the same was carried out in set 2 (UNFAIRE sample) except without the formaldehyde. Crosslinking was stopped by adding 2.5 ml 2M Glycine to every falcon (to a final concentration of 100 mM) and vacuum infiltration continued for 5 minutes. Plant material was rinsed 3 times with sterile water for 10 minutes under agitation at 4°C (by using falcon tubes), dried on sterile filter paper, and freeze fixed in liquid nitrogen.

Plant material was then ground to a fine powder and each sample was suspended in 30 ml **Buffer 1** and incubated on ice for 10-15 minutes. The suspension was filtered using Miracloth into a new pre-cooled falcon and centrifuged at 2880g for 20 minutes at 4°C. The pellet was suspended in 1 ml **Buffer 2**, transferred to an Eppendorf and centrifuged at 12000g for 10 minutes at 4°C. The washing with **Buffer 2** was repeated twice. The formed pellet was then suspended in 300 µl **Buffer 3** and overlaid on 300 µl ice-cold **buffer 3** in a fresh Eppendorf. This Eppendorf was centrifuged at 16000g for 70 minutes at 4°C. The chromatin pellet was suspended in 300 µl ice-cold **nuclei lysis buffer (NLB).** This was then sonicated at low power, pulses 15” on, 100” off for 10 times at 4°C. The sonicated chromatin was spun at 16000g for 10 minutes at 4°C. The supernatant was carefully transferred to a new tube to isolate nucleosome depleted regions (NDRs).

An equal amount (300 µl) of PCI (25:24:1) was added to the sample. It was vortexed and centrifuged at 12000g for 10 minutes at RT. The upper-aqueous phase containing NDRs was transferred to a new tube and the PCI step repeated twice to get highly pure sample. 0.1 volume of 3M sodium acetate (to a final concentration of 0.3M), 2.5 volume of absolute ethanol and 1 µl glycogen was added to the tubes and mixed well. The samples were stored at -20°C overnight.

DNA was pelleted at 16000g for 45 minutes at 4°C. The pellet was washed thrice with 1 ml 70% ethanol at 11000g for 7 minutes at RT. The supernatant was discarded, the pellet dried and eluted in 100 µl sterile water. This was then used for qPCR analyses.

Buffer 1

- 400 mM sucrose
- 10 mM Tris-HCl, pH 8
- 5 mM ß-mercaptoethanol (Fluka)
- 0.1 mM PMSF
- 1 tablet Complete Protease Inhibitor Cocktail per 50 ml buffer

Buffer 2

- 250 mM sucrose
- 10 mM Tris-HCl, pH 8
- 10 mM $MgCl_2$(Merck)
- 1% Triton X-100
- 5 mM ß-mercaptoethanol
- 0.1 mM PMSF
- ½ tablet Complete Protease Inhibitor Cocktail per 10 ml buffer (added just before use)

Buffer 3

- 1.7 M sucrose
- 100 mM Tris-HCl, pH 8
- 0.15% Triton X-100
- 2 mM $MgCl_2$
- 5 mM ß-mercaptoethanol
- 0.1 mM PMSF
- ½ tablet Complete Protease Inhibitor Cocktail per 10 ml buffer (added just before use)

Nuclear Lysis Buffer (NLB)

- 50 mM Tris-HCl, pH 8
- 10 mM EDTA
- 1% SDS
- 0.1 mM PMSF
- ½ tablet Complete Protease Inhibitor Cocktail per 10 ml buffer (added just before use)

2.12 Gene Expression Analysis using qRT-PCR

Real Time quantitative PCR experiments were performed on a Roche LightCycler 480 II using SYBR Green I master mix (Roche Applied Science). Each well contained 1.5µl of the isolated sample (1 µl in case of FAIRE sample), 0.5µl each of forward and reverse primers upto a concentration of 10µM, 6µl SYBR Green I and the volume made up to 12µl with autoclaved MQ water. ACTIN was used as the control gene. The analysis was done using MS-Excel.

The following primers were used for qPCR experiments:

GENE	SEQUENCE	REFERENCE
Actin F	CGTTTCGCTTTCCTTAGTGTTAGCT	
Actin R	AGCGAACGGATCTAGAGACTCACCTTG	
pFT 5'UTR F/ pFT F	GTTATGATTTCACCGACCCG	Castillejo and Pelaz, 2008
pFT 5'UTR R/ pFT R	GATCCAAGCCATTAGTCACC	Castillejo and Pelaz, 2008
pFT CORE 1&2 F	TAACTCGGGTCGGTGAAATC	Adrian et al., 2010
pFT CORE1&2 R	GTGGCTACCAAGTGGGAGAT	Adrian et al., 2010
pFT E-box F	GCTATATGCACTTTTTAACGACTAGC	Tripathi et al., 2017
pFT E-box R	CTGCGACTGCGACCTATTTT	Adrian et al., 2010
pFT CCAAT box F	TCTTGACATGGAGCGAAAGA	Adrian et al., 2010
pFT CCAAT box R	GGCCAACATTAGAAGAAGATTCC	Adrian et al., 2010
GA3ox1 F	TCTTCCAATCTCCCATCACC	Osnato et al., 2012
GA3ox1 R	TCCCGGAGAGATGTGAAGTC	Osnato et al., 2012
GA3ox2 F	CACTCCTCTTCTCCACCAAAA	Osnato et al., 2012
GA3ox2 R	CGTGTAAGAATCCGGGAGAG	Osnato et al., 2012

3. OBJECTIVES

The main objective of this thesis was to shed some light on molecular interactions occurring during floral repression in *Arabidopsis thaliana*. To achieve this, we decided to use several molecular biology techniques including ChIP and FAIRE to study the various aspects of the relationship between flowering repressors TEM and MYC.

The specific goals were as follows:

1. To analyse histone mark levels on *FT* promoter in *tem*, *myc* and *myc tem* mutant backgrounds. We attempt to uncover if the TEM/MYC repression of *FT* occurs at the histone modification level.
2. To analyse nucleosome occupancy in the *FT* promoter region in the same mutant backgrounds, to unravel a putative function of TEM and MYC in chromatin remodelling.
3. To analyse the interplay between TEM and MYC to explore the necessity of their interaction for the target genes binding.

4. RESULTS

4.1 Analysing expression levels of histone modification marks on *FT* promoter regions

TEMs and MYCs are proven flowering repressors by directly binding regulatory sequences of *FT*, accordingly their mutants have an early flowering phenotype (Castillejo and Pelaz, 2008; Osnato et al., 2012, Wang et al., 2017, Bao et al., 2019). Our lab also found that TEM and MYC proteins interact in yeast 2-hybrid and Bi-FC assays, and created a quintuple mutant with a combination of both, having *tem1, tem2, myc2, myc3* and *myc4* mutations. The quintuple *myc2 myc3 myc4 tem1 tem2* mutant (hereafter, *myc tem*) ended up flowering much earlier than the double *tem1 tem2* (hereafter *tem*) or triple *myc2myc3myc4* (hereafter *myc*) mutants; which corresponded with higher levels of *FT* expression (Osnato and Pelaz unpublished). The fact that TEMs and MYCs were found to interact with TPL (Causier et al., 2012, Chini et al., 2009; Niu et al., 2011; Fernandez-Calvo et al., 2011), and TEMs also interact with the histone methyltransferase CURLY LEAF (CLF*)* in plants (Hu et al., 2021), suggested that protein complexes of which TEM and MYC are part could recruit histone modification enzymes for the regulation of *FT*.

Histone acetylation is usually associated with increased gene transcription (Kuo et al., 1998; Reid et al., 2001). In this epigenetic modification, a negatively charged acetyl group is usually added to a lysine on the histone tail. It is theorised that changes in the charge of the amino acid, lysine, an addition of the acetyl group, make the chromatin more readily accessible to transcription factors and RNA polymerase (Marushige, 1976; Lee et al., 1993; Vettese-Dadey et al., 1996; Mashumoto et al., 2005; Shahbazian and Grundstein, 2007). The levels of histone acetylation on genes are balanced by the opposing actions of HATs and HDACs.

Histone lysine methylation, on the other hand, can repress or activate a gene, depending on the location of the lysine residue and the number of methyl groups added to it. Methylation can occur on all basic amino acids in histones including lysines and arginines (Murray, 1964; Byvoet et al.1972). This happens when one, two or three methyl groups are added to the lysine by histone lysine methyltransferases (Murray, 1965; Paik and Kim, 1967; Haempel

et al., 1968). Methyltransferases, unlike acetyltransferases, are very specific to their respective lysine residues (Bannister and Kouzarides, 2005). H3K27me is associated with gene repression. In *Arabidopsis*, H3K27me3 is largely restricted to transcribed regions of a gene, like the promoters including the 5'UTR (Zhang et al., 2007).

As discussed earlier, HDA6 and HDACs (through TPLs) have been implicated to have a role in floral regulation. Direct interactions of TEMs with TPL and MYC suggest that histone modifications might be responsible, at least in part, for the early flowering phenotypes of the *tem* and *myc* mutants. Since most post-transcriptional chromatin modifications occur on specific regions of target genes like the upstream region of the promoter, the core promoter, the 5'UTR or the 3'UTR, we chose the four main regulatory regions of the *FT* promoter, for our analyses. We chose, from proximal to distal to the *FT* ATG, the 5'UTR region where TEMs and MYC2 bind, CORE 1 and 2 which are key for *FT* activation as CO is brought here by interacting with NF-Y proteins, an E-box that could be target of MYC proteins and a distal CAATC region where the NF-Y proteins bind.

We did ChIP experiments to isolate H3 acetylated and H3K27me3 chromatin followed by qPCRs to study the expression levels of acetylated and methylated chromatin in the different mutants under LD and SD. Although we performed several biological replicates, they resulted in high biological variability which could be attributed to the different growth chambers or different light quality as the institute was changing all light bulbs to LEDS. There were also different growth conditions in different chambers and we observed a lot of fluctuations during the growth period multiple times. We present the last two replicates performed in the same light quality but still with biological variability.

We first grew plants of the four genotypes, Col, *tem*, *myc* and *myc tem*, under LD, collected seedlings 7 days after germination at ZT16. As we can see in figures 6 and 7, the levels of H3 acetylation in the 4 genotypes under LD do not follow a specific trend corresponding to the phenotypes observed. In the first replicate, Col and *tem* plants showed higher acetylation among the genotypes tested in all four regions tested (Figure 6) whereas in the second replicate, the higher acetylation was maintained in the 5'UTR of Col and *tem* in the CORE region of the *tem* mutants (Figure 7).

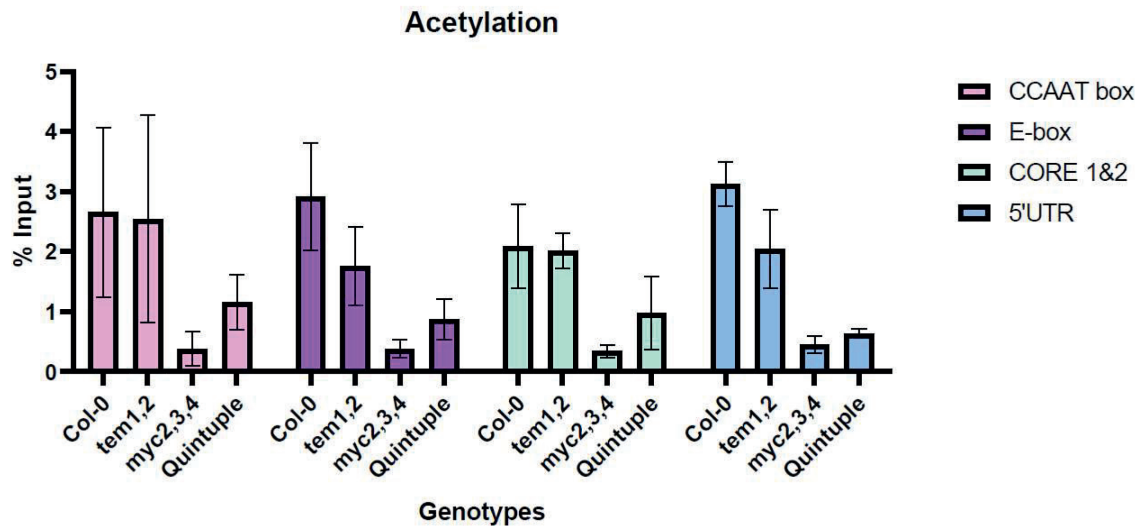


Figure 6: Histone Acetylation (LD) Replicate 1. 7-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT16 and ChIP performed using anti-H3Ac. qPCRs were performed using primers specific to the four FT promoter regulatory regions presented in the order they appear on the promoter from farther upstream to closer to the ATG. The bars represent %input of H3 acetylation of the sample genotype normalized using no antibody and actin as control.

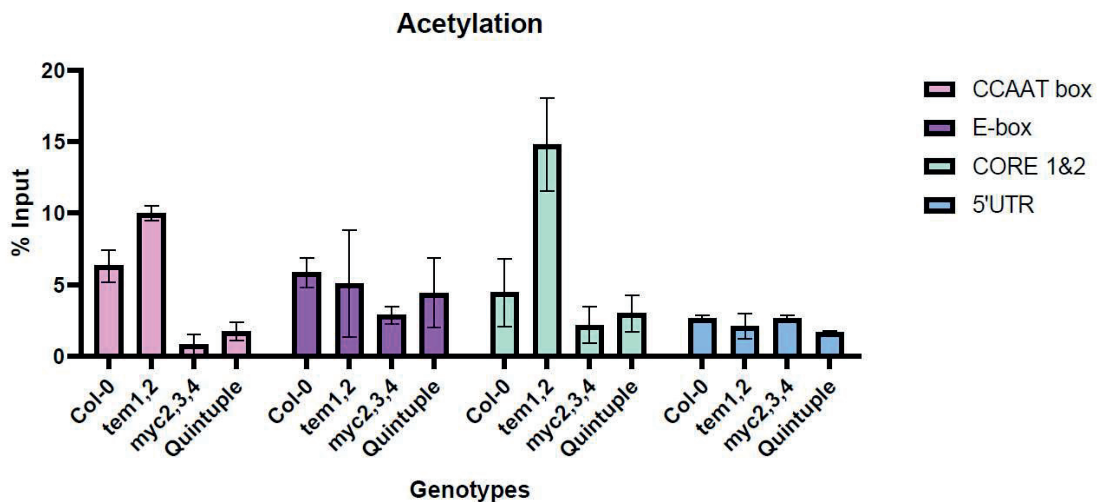


Figure 7: Histone Acetylation (LD) Replicate 2

Similarly, when we performed the experiment in plants grown under SD and collected tissue 10 days after germination at ZT8, we found that in the first replicate Col control plants, which flower the latest among the four genotypes, showed higher acetylation (Figure 8),

whereas in the second replicate the CORE region of the *tem* mutants showed the highest levels of histone acetylation (Figure 9). As acetylation is largely an activation mark, the quintuple mutant should theoretically have higher levels of acetylation as several repressors from the flowering pathway are missing in this background, which results in higher *FT* expression levels and it is in line with the early flowering phenotype we observed.

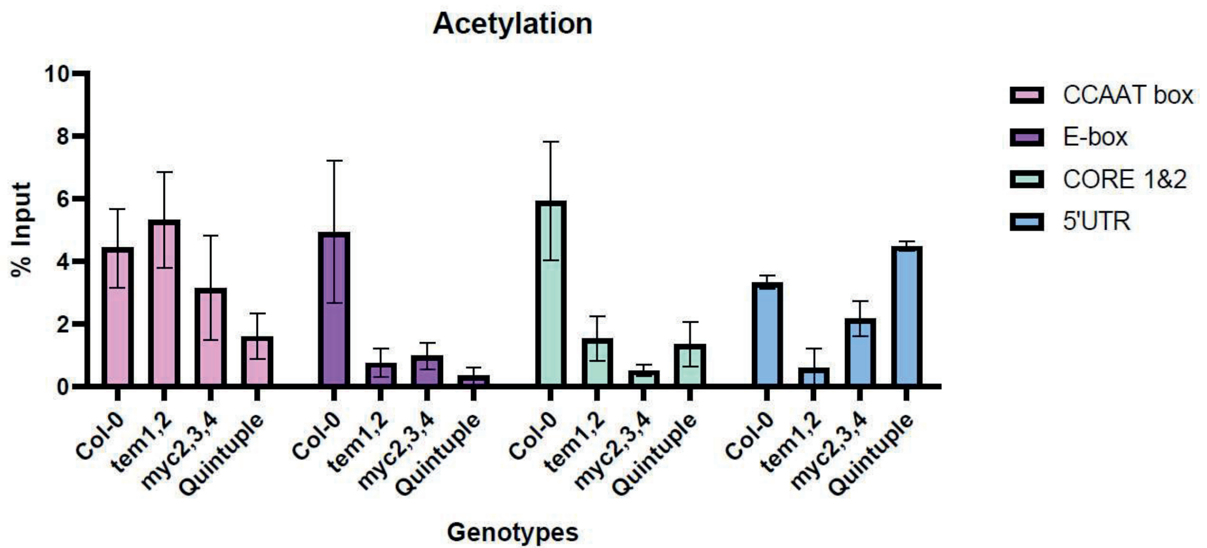


Figure 8: Histone Acetylation (SD) Replicate 1. 10-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT8 and ChIP performed using anti-H3Ac. qPCRs were performed using primers specific to the four *FT* promoter regulatory regions. The bars represent %input of the sample genotype normalized using no antibody and actin as control.

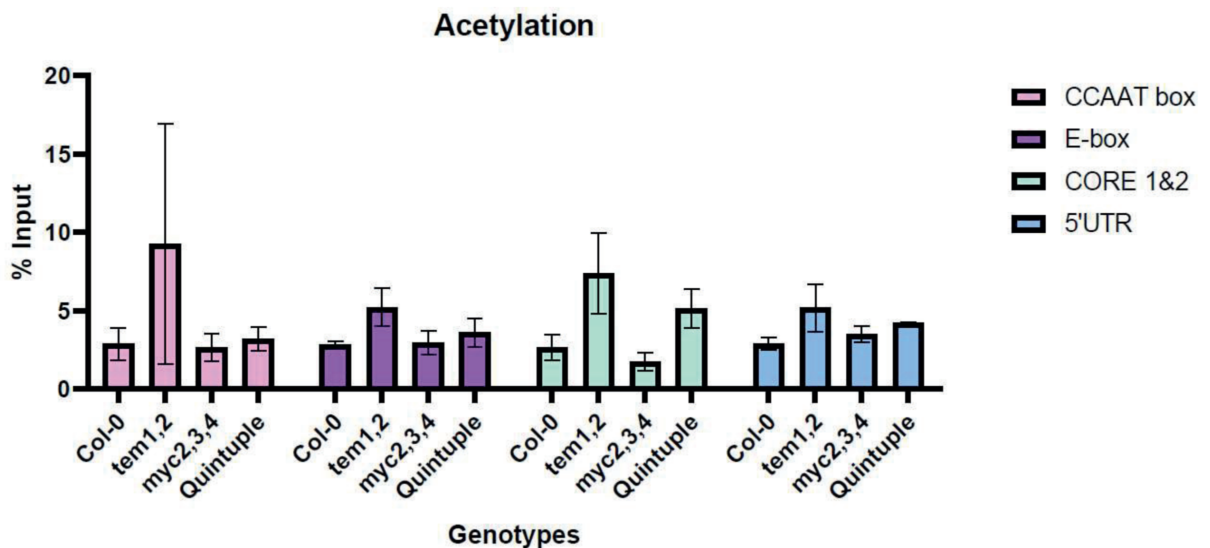


Figure 9: Histone Acetylation (SD) Replicate 2

In summary, we conclude that there are no significant changes in the levels of acetylation in the *FT* promoter regions of the mutants compared to the wild type plants that could explain the differences in their flowering times.

We then measured the levels of tri-methylation of Lysine on H3, H3K27me3, a repressive mark in the same four regions of the same four genotypes. Like done previously, plants were grown under LD and tissue collected at 7 days after germination at ZT16. We observed that the *myc* mutant had a higher level of methylation compared to the *tem* or the *myc tem* mutants in all the regions of the *FT* promoter, which correlates with the *myc* phenotype being slightly later than the *tem* or *myc tem* ones (Figures 10 and 11). However, we did not observe this trend in the Col-0 (the control) which has the latest flowering time of all genotypes (Figure 10) although in the second replicate the levels of methylation of Col plants are comparable to those of *myc* mutants (Figure 11) in the 5'UTR, CORE 1&2 and the CCAAT box elements.

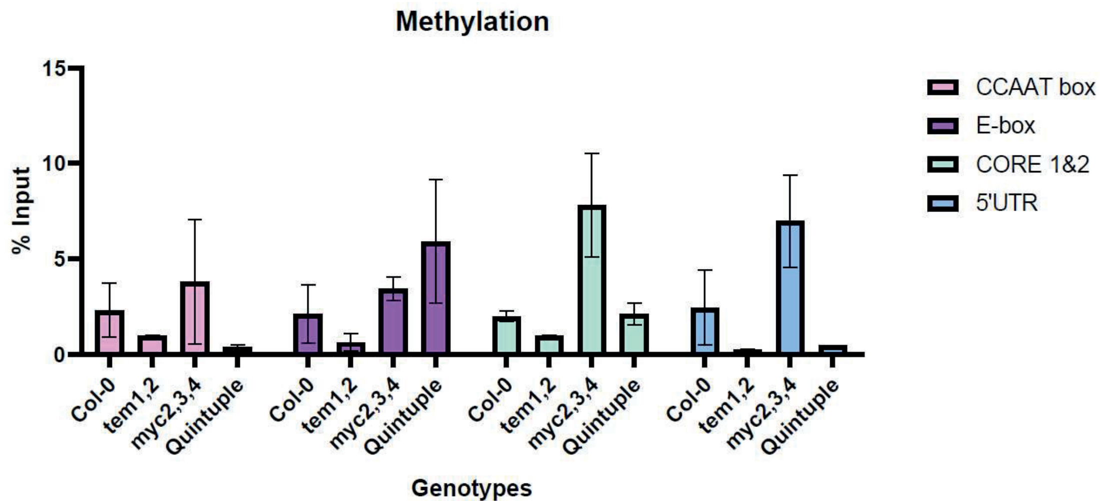


Figure 10: Histone Methylation (LD) Replicate 1. 7-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT16 and ChIP performed using anti-H3K27me3. qPCRs were performed using primers specific to the four FT promoter regulatory regions presented in the order they appear on the promoter from farther upstream to closer to the ATG. The bars represent %input of H3 lysine trimethylation of the sample genotype normalized using no antibody and actin as control.

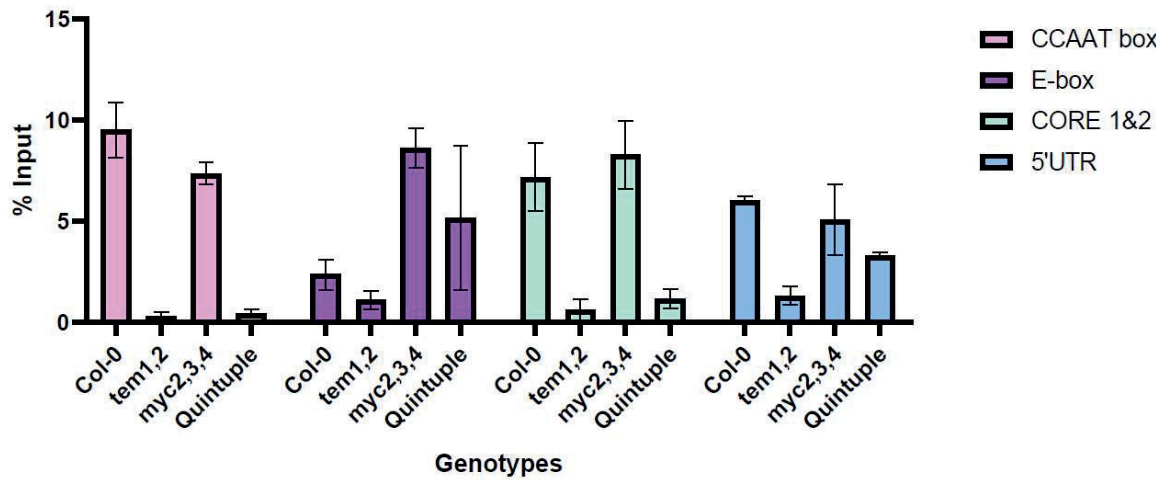


Figure 11: Histone Methylation (LD) Replicate 2

We then grew the four genotypes under SD and collected the tissue 10 days after germination at ZT8. In figure 12, we observe that *myc* and *tem* have lower levels of methylation than Col, which is plausible because they flower earlier than Col and will theoretically have lower levels of a repressive mark, although this does not explain the higher methylation in the quintuple mutant which flowers earliest of them all. In figure 13, we can see that the *tem* mutant has a higher level of methylation than the rest of the genotypes in the regulatory regions of the *FT* promoter under SD. As H3K27me3 is a repressive mark, it would be rational to assume that the *tem* mutant would have a higher amount of it compared to the quintuple mutant but not compared to Col-0, as the levels of H3K27me3 of *tem* are similar to Col-0.

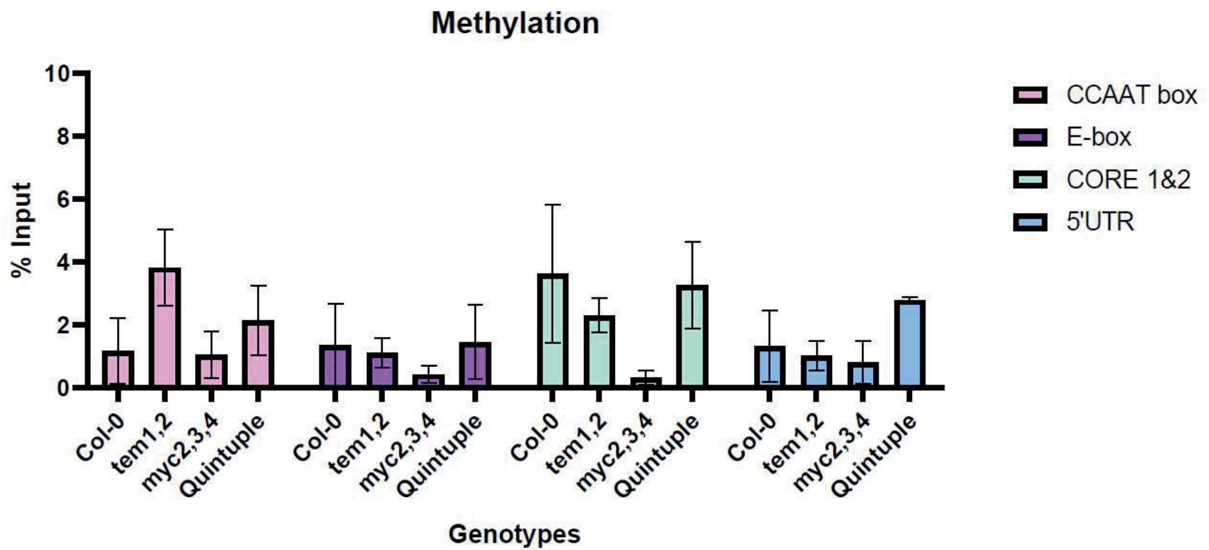


Figure 12: Histone Methylation (SD) Replicate 1. 10-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT8 and ChIP performed using anti-H3K27me3. qPCRs were performed using primers specific to the four FT promoter regulatory regions. The bars represent %input of the sample genotype normalized using no antibody and actin as control.

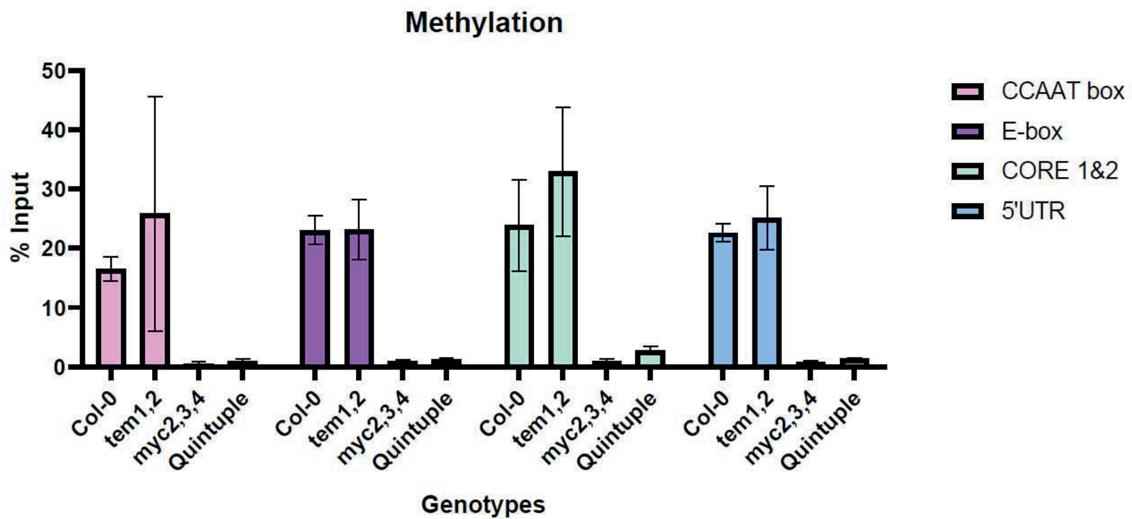


Figure 13: Histone Methylation (SD) Replicate 2

Overall, we can say that the histone marks analyses did not give any conclusive results in proving that this could be the reason behind the differences in the flowering time phenotypes observed. The results were very variable in all conditions due to a number of reasons and cannot be used to definitely prove that the TEM and MYC interactions are responsible for modifying histone marks, and thereby affecting transcription, in the studied region of the *FT* promoter.

4.2 Analysing the effect on Nucleosome occupancy in *FT* promoter regions

As mentioned in the introduction, the CO/NF-Y complex and ultimately the formation of the *FT* promoter loop is essential for *FT* transcription initiation. CO binding to the *FT* promoter leads to transcription activation (Suárez-López et al., 2001, Valverde et al., 2005) whereas TEM binding at a nearby site leads to transcription repression (Castillejo and Pelaz, 2008, Osnato et al., 2012). One possibility how this occurs could be that TEM binding to the *FT* promoter somehow hinders CO binding or that TEM competes with CO to bind to the *FT* promoter. Similarly, it has been shown that MYC2 and MYC3 bind to different regions of the *FT* promoter and could prevent CO activity (Wang et al., 2017, Bao et al., 2019). It has been proposed, that under SD, MYC3 is stabilized by DELLA due to the low levels of GA and binds to the *FT* promoter which prevents the binding of the CO, present in low levels, and this is associated with compact chromatin at the region. Under LD, MYC3 binding is released by GA which allows the higher CO levels to bind *FT* when the chromatin is open (Bao et al., 2019).

To test the chromatin state of *tem*, *myc* and *myc tem* mutants compared with Col-0 plants, we decided to perform Formaldehyde Associated Isolation of Regulatory Elements (FAIRE) experiments, followed by qPCR. FAIRE is a technique used to separate histone bound (closed or heterochromatic) regions of the DNA from unbound or free (open or euchromatic) regions (Giresi et al. 2007; Louwers et al. 2009; Gaulton et al. 2010; Song et al. 2011b). This method allowed us to separate DNA which is being transcribed from inaccessible regions which are not available to TFs or RNA polymerase binding. Like in the histone mark analyses experiments, we grew the four genotypes under LD and collected between 50 and 70 seedlings 7 days after germination at ZT16.

As we can see in the graphs (Figures 14 and 15), no appreciable difference was found in the bound vs unbound DNA ratio among the different genotypes tested under LD. This result was unexpected because higher expression levels of a gene are oftentimes linked to the gene becoming more accessible. A possible reason why we could not detect any discernible differences in this ratio could be that under these conditions the chromatin is already open enough that it cannot unravel any more, and therefore, another factor is responsible for the different phenotypes under LD. It could also be that the nucleosome occupancy is affected in a region other than the four *FT* promoter elements we checked.

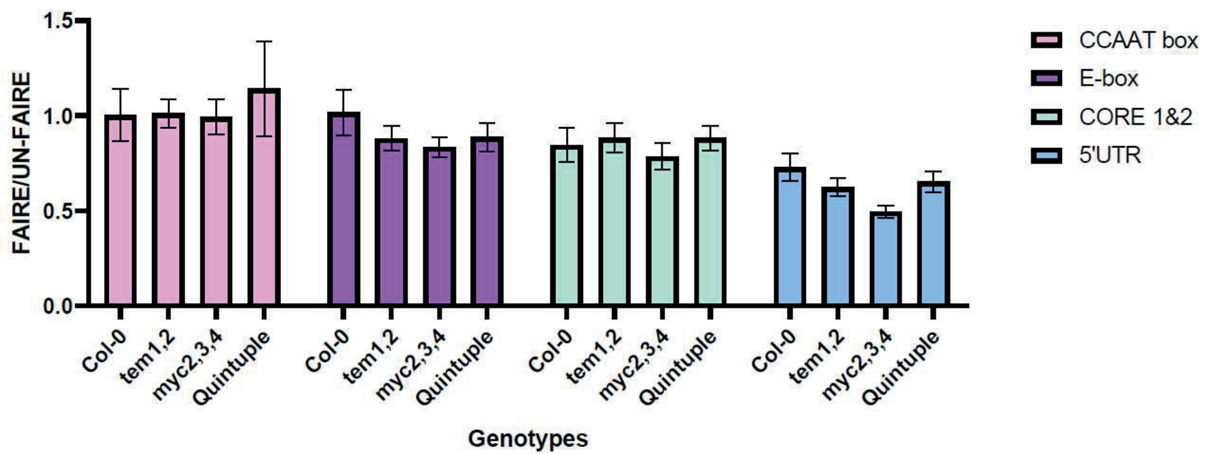


Figure 14: FAIRE (LD) Replicate 1. 7-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT16 and their DNA-protein interactions fixed using formaldehyde. FAIRE followed by qPCRs was performed using primers specific to the four FT promoter regulatory regions presented in the order they appear on the promoter from farther upstream to closer to the ATG. The bars represent the ratio of bound vs unbound DNA in the tested region.

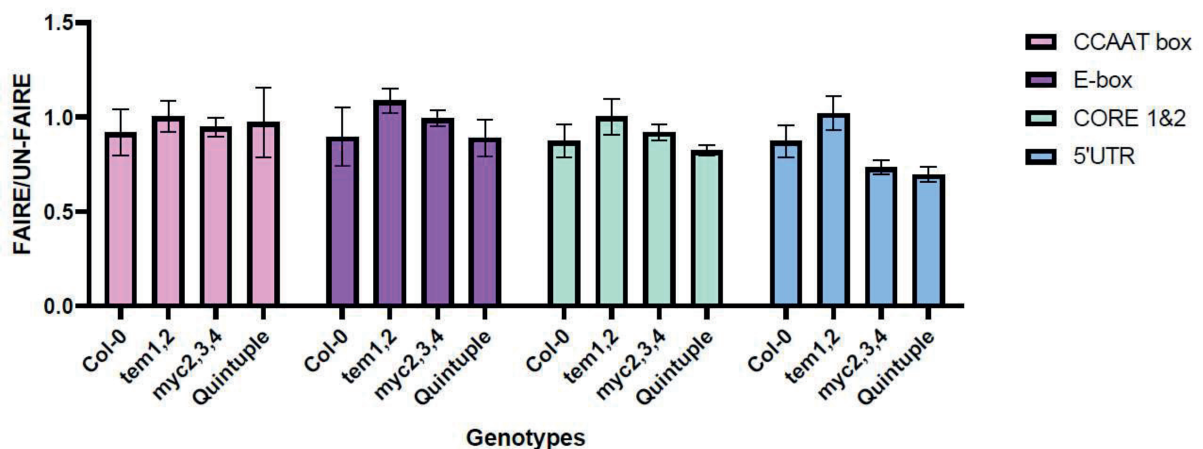


Figure 15: FAIRE (LD) Replicate 2

Under SD, however, the quintuple mutant had a higher amount of free DNA compared to Col-0 in almost all the regions (Figures 16, 17). The quintuple mutant is the earliest flowering mutant of all and had a higher amount of unbound DNA in the CORE 1&2 elements of the *FT* promoter, which is the region where CO binds. These differences could explain the early flowering phenotype of this mutant under SD. Although this difference

could not be found in LD samples, there may be different mechanisms involved in *FT* regulation in the two photoperiod conditions.

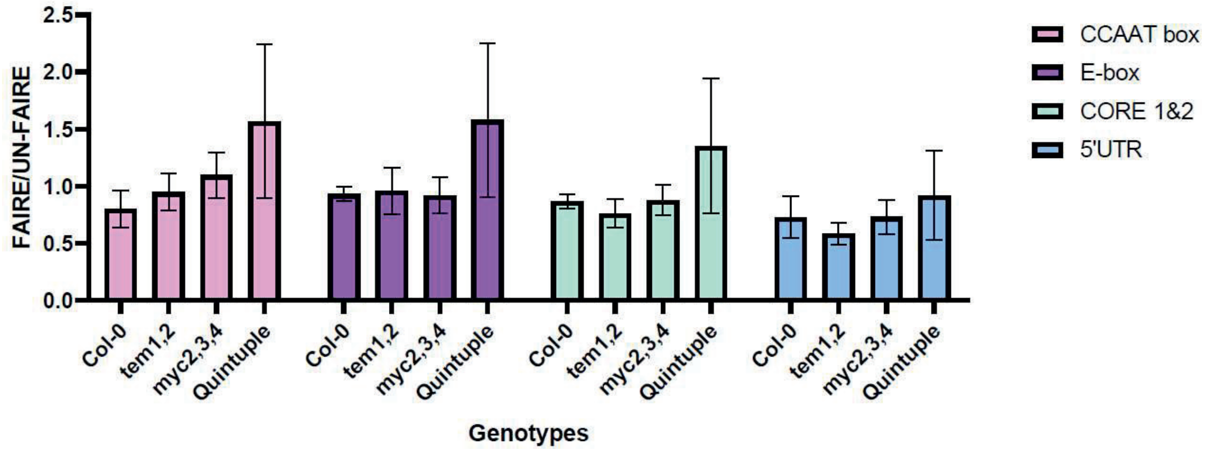


Figure 16: FAIRE (SD) Replicate 1. 10-day old Col-0; *tem; myc* and quintuple mutant seedlings were harvested at ZT8 and FAIRE performed. qPCRs were done using primers specific to the four FT promoter regulatory regions. The bars represent the ratio of bound vs unbound DNA in the sample genotypes.

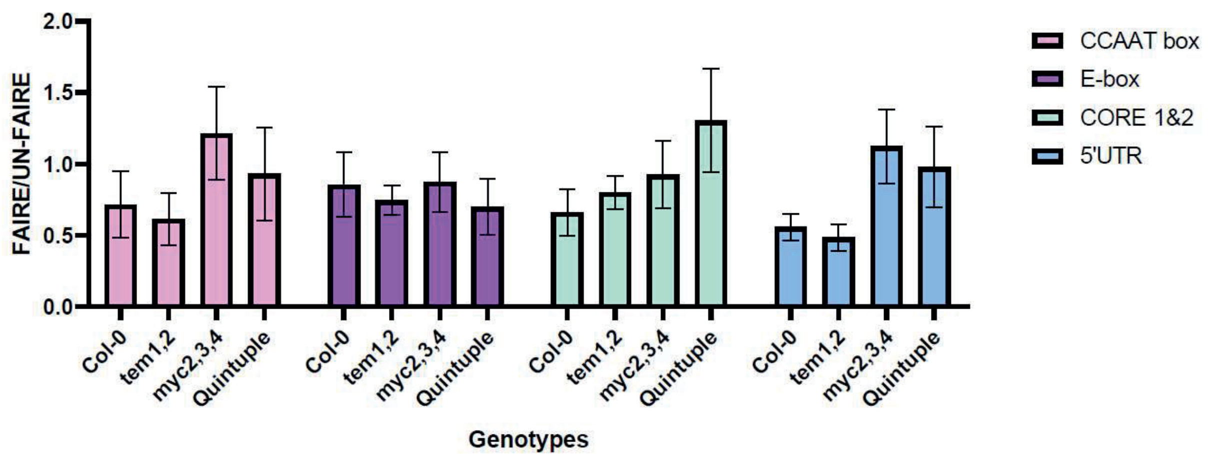


Figure 17: FAIRE (SD) Replicate 2

4.3 Analysing the Existence/Requirement of a TEM/MYC complex

TEM and MYC are both transcriptional repressors of *FT* and bind to the same region of the *FT* promoter delaying flowering. Y2H studies have also shown that TEM and MYC interact (Osnato and Pelaz unpublished). Interestingly, they share other common direct or indirect, interactors such as TPL (Causier et al., 2012, Pauwels et al., 2010) which in turn acts with the aid of histone modifiers (Long et al., 2006). Even though TEM and MYC interact and have common targets, the quintuple (*myc tem*) mutant showed a more pronounced early flowering phenotype with higher amounts of FT compared with *tem* and *myc* mutants. This suggests that although they have common targets in the flowering pathway, they may also have independent targets responsible for the quintuple mutant phenotype.

Although they have *FT* as a common target, we wondered if the TEM/MYC protein interaction is required for their binding. To check whether TEM and MYC form a complex to affect their independent and common targets, we decided to perform ChIP-qPCR experiments to detect differences in the binding of TEM and MYC to their target sites in the presence or absence of each other.

We designed a *TEM1* cDNA construct under the control of its putative endogenous promoter tagged to HA, transformed it into WT (*Arabidopsis thaliana* Col-0) and *tem* mutants to obtain stable homozygous transformant lines. We obtained several independent transgenic lines and selected 6 to 10 for further characterization. We performed flowering time experiments under LD and SD. The transgenic lines in the Col background did not result in changes in flowering time, only two lines, D5 and E3, showed a very slight late flowering phenotype, if any, both under LD and SD (Figures 18 and 20). This does not seem strange as the increase in the TEM1 content over the endogenous levels might not be relevant. In the *tem* mutant background there was little to no recovery of the mutant phenotype by the construct except in one line, i.e., J.3 (Figure 19). The line J.3 had other visible differences with the WT like absence of petioles, a very compact rosette structure and unusual leaf shape. This indicated that the insertion site might have had an effect in this line unrelated to the T-DNA inserted.

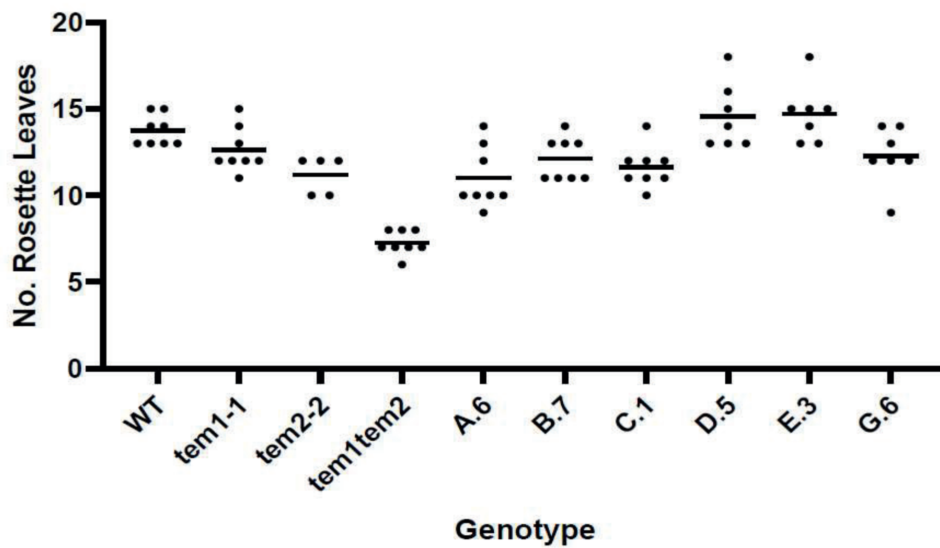


Figure 18: Flowering Time in LD – pTEM1::HA::TEM1 in WT. We chose 6 independent lines A.6, B.7, C.1, D.5, E.3 and G.6 for the experiment. Each dot represents number of rosette leaves in individual plants.

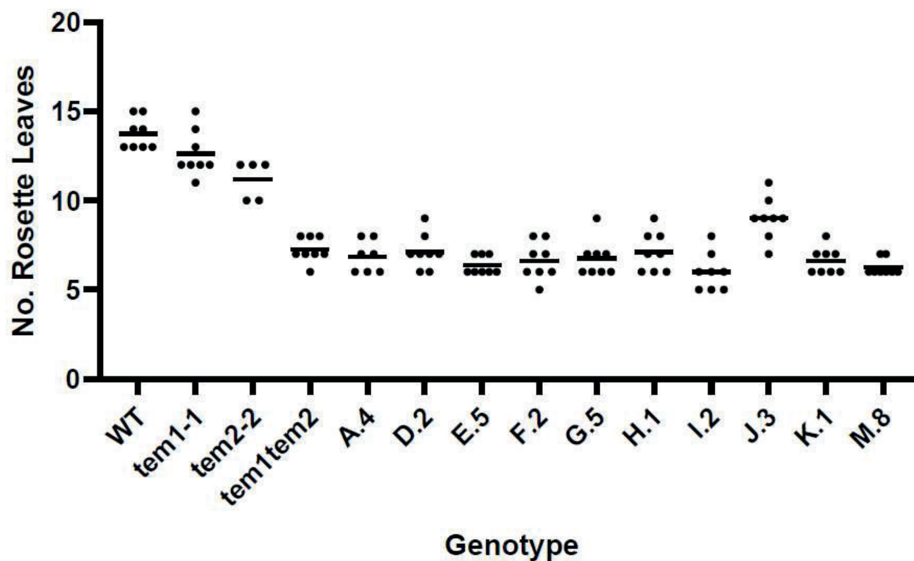


Figure 19: Flowering Time under LD – pTEM1::HA::TEM1 in *tem1tem2*. 10 independent lines were chosen for analysis and their rosette leaf number counted. Each dot represents number of rosette leaves in individual plants.

Under SD, the results were similar in terms of mutant phenotype recovery. The *tem* mutant background, as well as the transgenic lines, showed a high variability in the number of

rosette leaves on flowering but the mean of the values still did not delay flowering compared to the control (Figure 21).

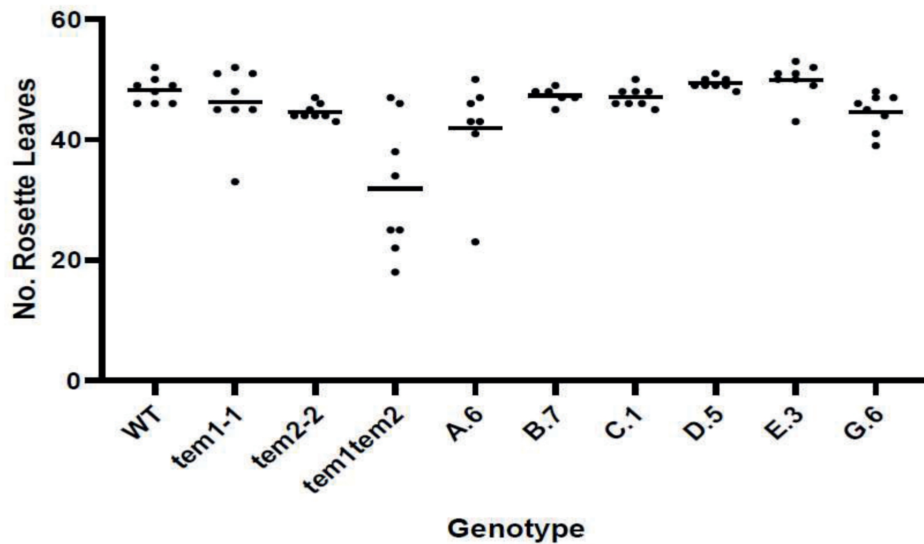


Figure 20: Flowering Time under SD – pTEM1::HA::TEM1 in WT. Rosette leaf number of the 6 independent lines was counted. Each dot represents number of rosette leaves in individual plants.

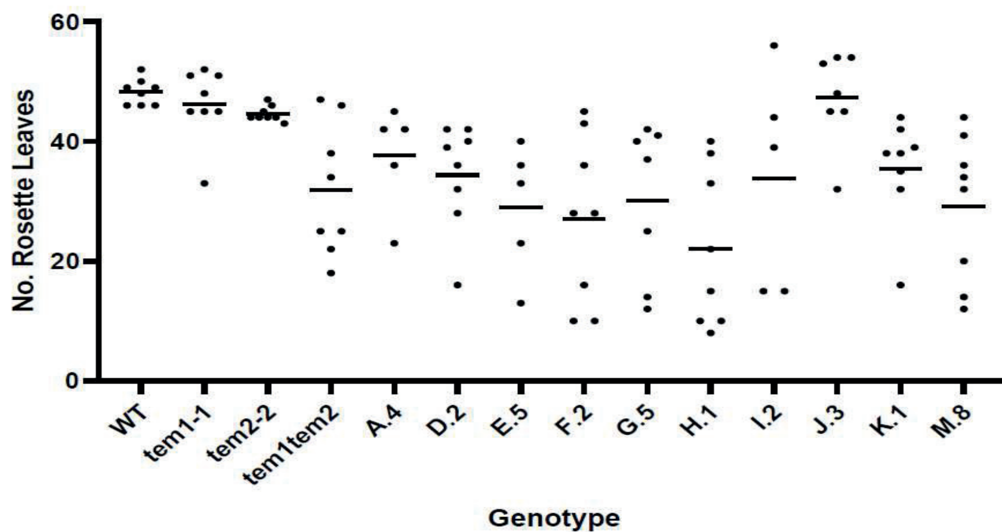


Figure 21: Flowering Time under SD – pTEM1::HA::TEM1 in tem1tem2. Rosette leaf number of the 10 independent lines was counted. Each dot represents number of rosette leaves in individual plants.

Assuming that a mistake in the *TEM1* promoter prediction might be responsible for the lack of mutant phenotype recovery in the mutant backgrounds, we decided to switch strategies and go with tissue specific promoters pKNAT1 and pSUC2 (expressed in the shoot apical

meristem and phloem respectively) but followed the same concept. The lab had untagged pKNAT1::TEM2 and pSUC2::TEM2 lines in Col background available, created by a previous lab member. We decided to check the flowering time phenotype of these transgenic lines under LD to test the appropriateness of these promoters. We observed a very strong delay of the flowering time in the lines with pKNAT1::TEM2 or pSUC2::TEM2. Wild type plants flowered after the formation of around 12 rosette leaves whereas pKNAT1::TEM2 and pSUC2::TEM2 lines flowered after producing on average 15 to 20 rosette leaves (Figures 22 and 23).Therefore, we decided to proceed with the strategy of using these specific promoters for our experiments.

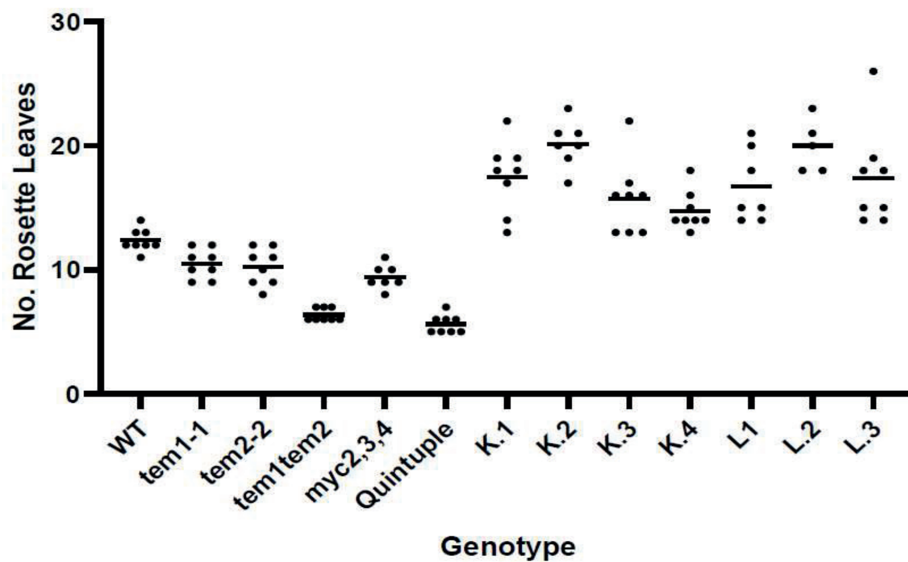


Figure 22: Flowering Time – pKNAT1::TEM2 in WT. Rosette leaf number of the 7 independent lines was counted and compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

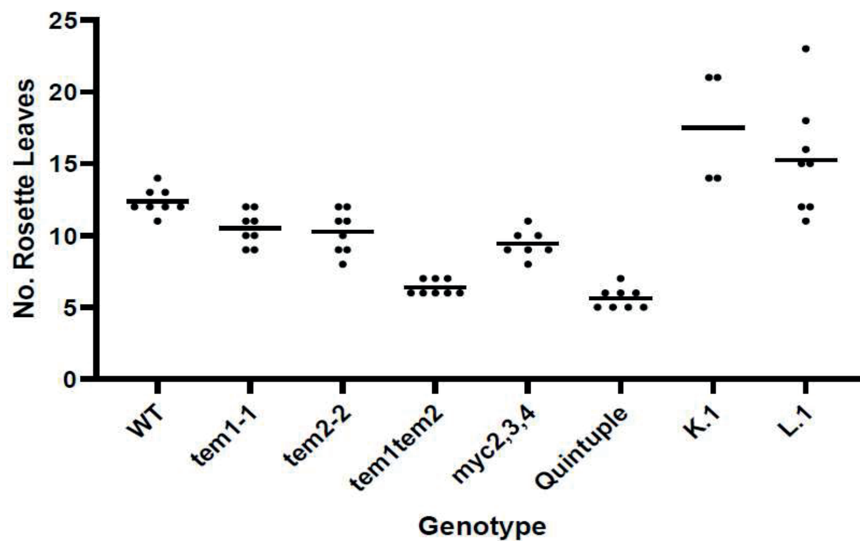


Figure 23: Flowering Time – pSUC2::TEM2 in WT. Rosette leaf number of 2 independent lines was counted and compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

We then generated tagged *TEM1* and *TEM2* constructs under the control of *pKNAT1* and *pSUC2*, resulting in pKNAT1::HA::TEM1, pKNAT1::HA::TEM2, pSUC::HA::TEM1 and pSUC::HA::TEM2. We used these constructs to transform WT, *tem*, *myc* and quintuple mutant genotypes and obtained homozygous lines. Transforming the quintuple mutant proved to be very difficult either due to the genetic background or the specific constructs. After multiple rounds of plant transformation, we managed to obtain T1 transformed seeds. GFP expression in the seeds was used to select transformed seeds as the plasmid bears the promoter of a seed-specific gene (*At2S3*) fused to GFP as marker, and all quintuple transformed lines lost their GFP phenotype by the T2 or T3 generation. Even though having this transformation would have made the study more comprehensive, we decided to proceed without it due to lack of time.

We obtained homozygous transformant lines in the WT, *tem* and *myc* backgrounds and performed flowering time experiments in T3 plants. Similar to the results obtained with the pTEM1::HA::TEM1 constructs, pKNAT1::HA::TEM1 and pKNAT1::HA::TEM2 transgenic lines showed a flowering time to comparable to or an even earlier flowering time than the Col-0 WT plants, although we found more variability among the transgenic lines (Figure 24, 26).

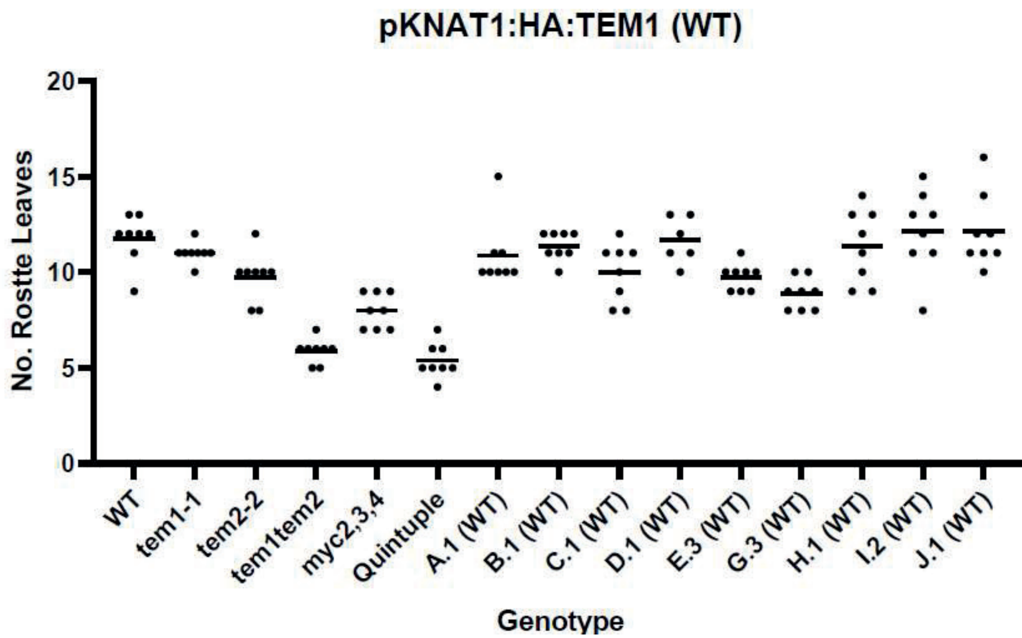


Figure 24: Flowering Time in LD – pKNAT1::HA::TEM1. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

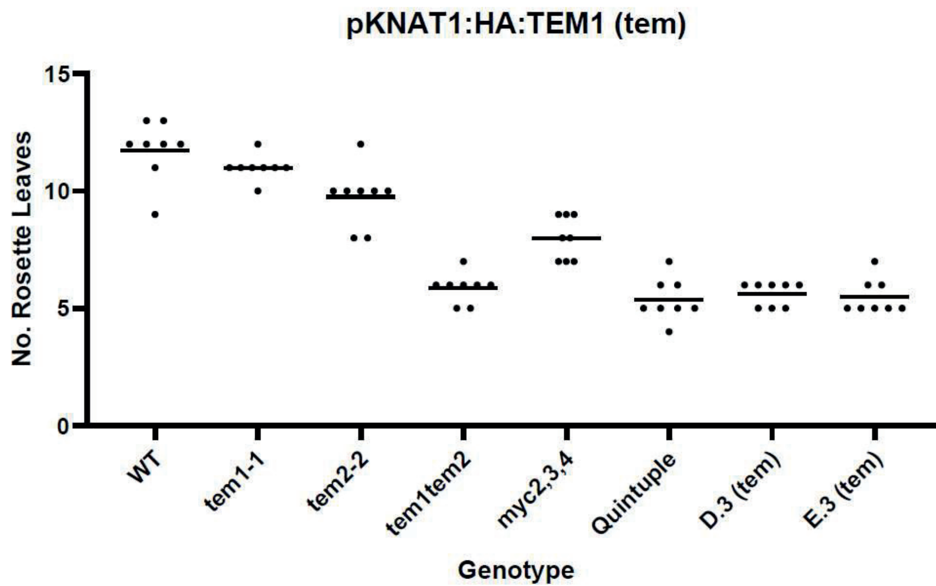


Figure 25: Flowering Time in LD – *tem* pKNAT1::HA::TEM1. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

Surprisingly, we observed important differences in the degree of the phenotype recovery in the mutant backgrounds with the tagged constructs with either promoter or either *TEM* gene.

We only found slight reduction on the *myc* mutant phenotype in pKNAT1::HA::TEM2 C4 and D1 lines (Figure 28) but there was no delay observed in lines in the *tem* backgrounds expressing either *TEM1* or *TEM2* (Figures 25 and 27) .

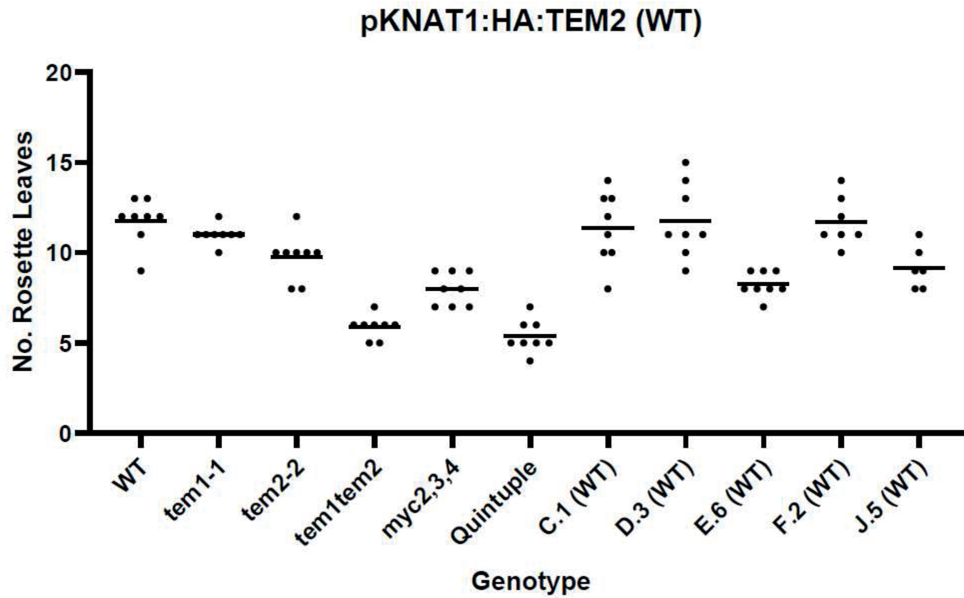


Figure 26: Flowering Time in LD – pKNAT1::HA::TEM2. Rosette leaf number of the independent lines compared with wt and mutant plants. Each dot represents number of rosette leaves in individual plants.

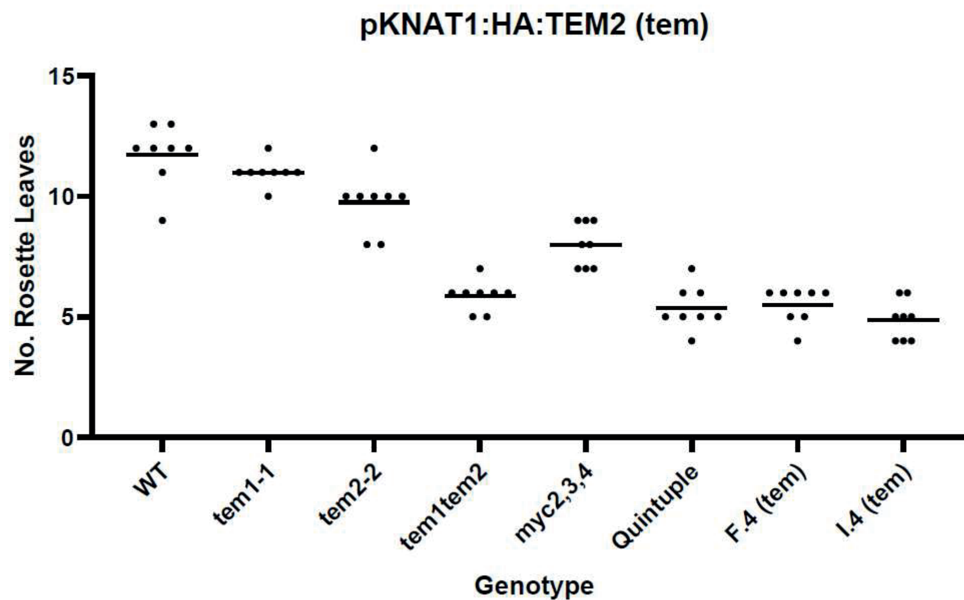


Figure 27: Flowering Time in LD – *tem* pKNAT1::HA::TEM2. Rosette leaf number of independent lines compared with wt and mutant plants. Each dot represents number of rosette leaves in individual plants.

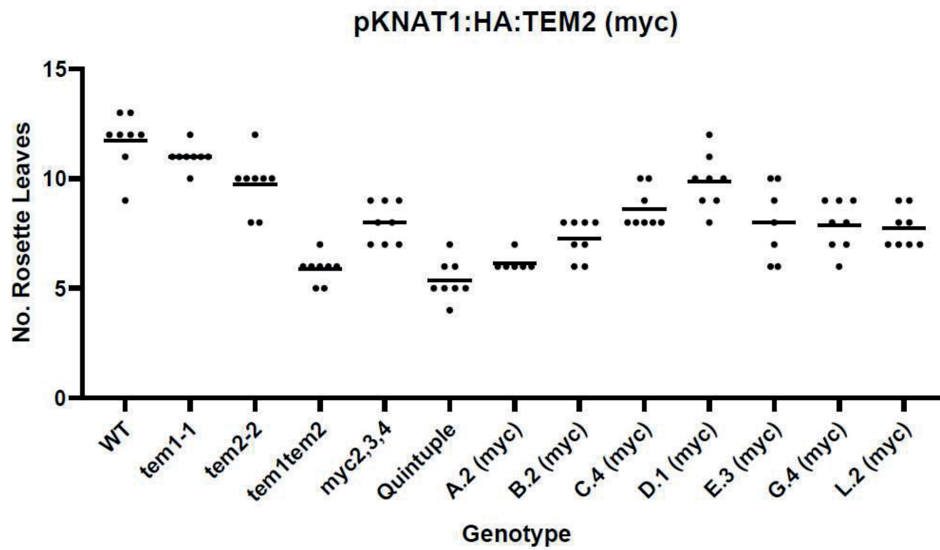


Figure 28: Flowering Time in LD – *myc* pKNAT1::HA::TEM2. Rosette leaf number of independent lines compared with wt and mutant plants. Each dot represents number of rosette leaves in individual plants.

Analogous to the phenotypes of lines expressing pKNAT1::HA::TEM, plants carrying pSUC2::HA::TEM1 construct did not show changes in their flowering time (Figures 29-31). Transgenic lines bearing the *pSUC2* promoter exhibited more promising results compared to *pKNAT1* simply because of their delay in flowering time. This is the case of pSUC2::HA::TEM1 in *tem1tem2*, lines R.3 and Q.2 (Figure 30) and pSUC2::HA::TEM1 in *myc2myc3myc4* line L.1 (Figure 31).

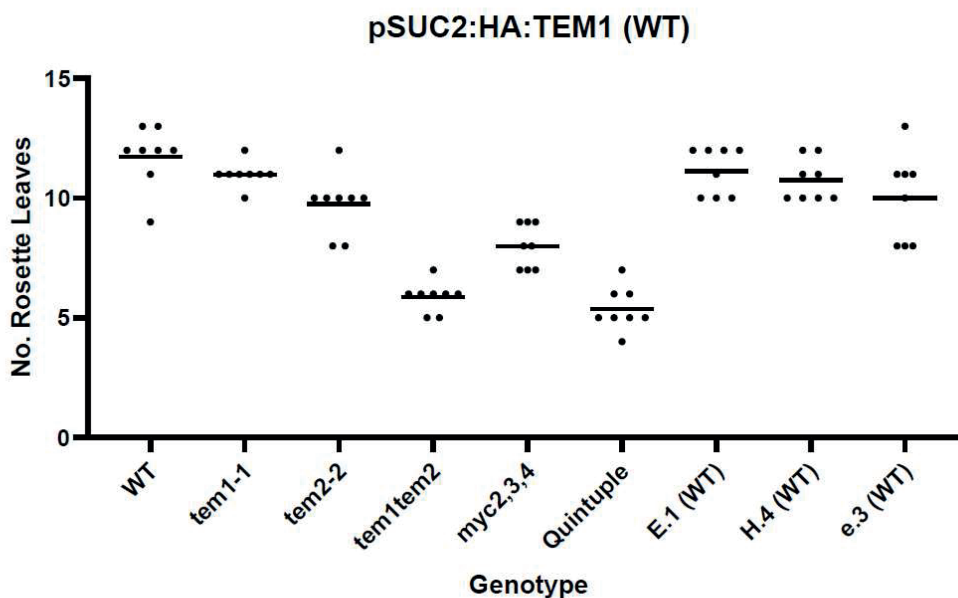


Figure 29: Flowering Time in LD – WT pSUC2::HA::TEM1. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

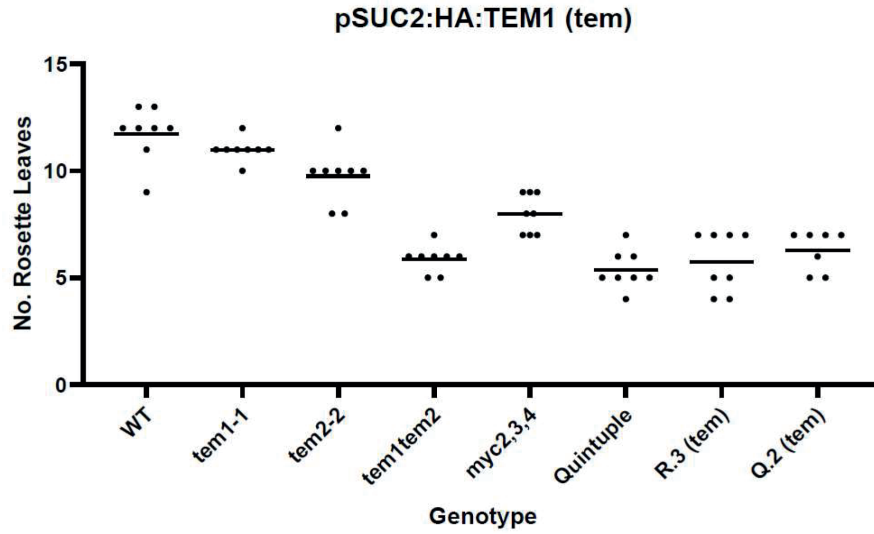


Figure 30: Flowering Time in LD – *tem* pSUC2::HA::TEM1. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

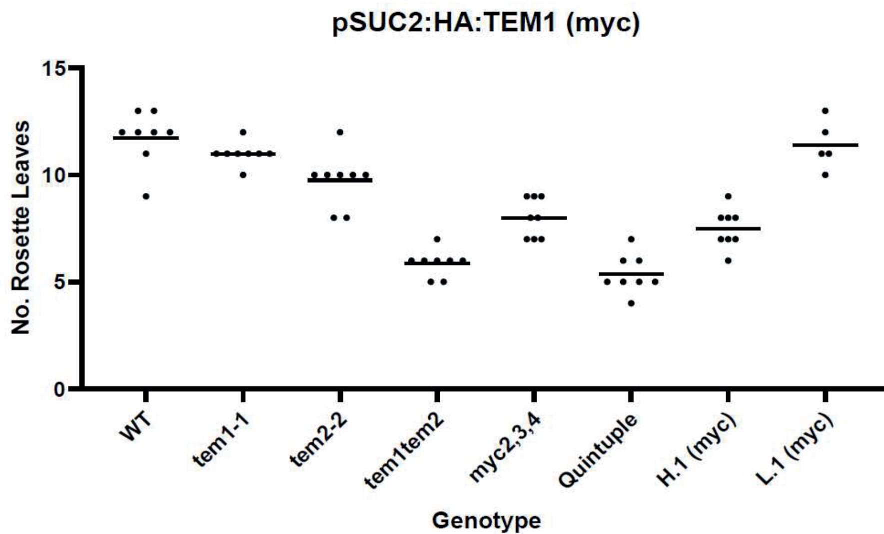


Figure 31: Flowering Time in LD – *myc* pSUC2::HA::TEM1. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

However several pSUC2::HA::TEM2 lines in the WT background, including B.1, C.1, E.2 and G.2, displayed a late flowering phenotype when compared to WT plants (Figure 32).

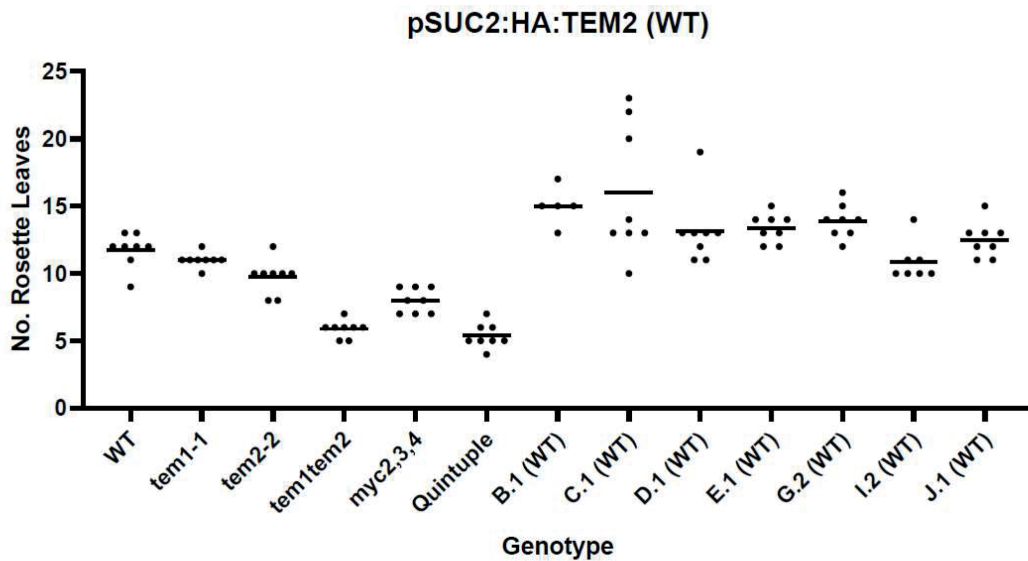


Figure 32: Flowering Time in LD – WT pSUC2::HA::TEM2. Rosette leaf number of independent lines compared with wt and mutant plants. Each dot represents number of rosette leaves in individual plants.

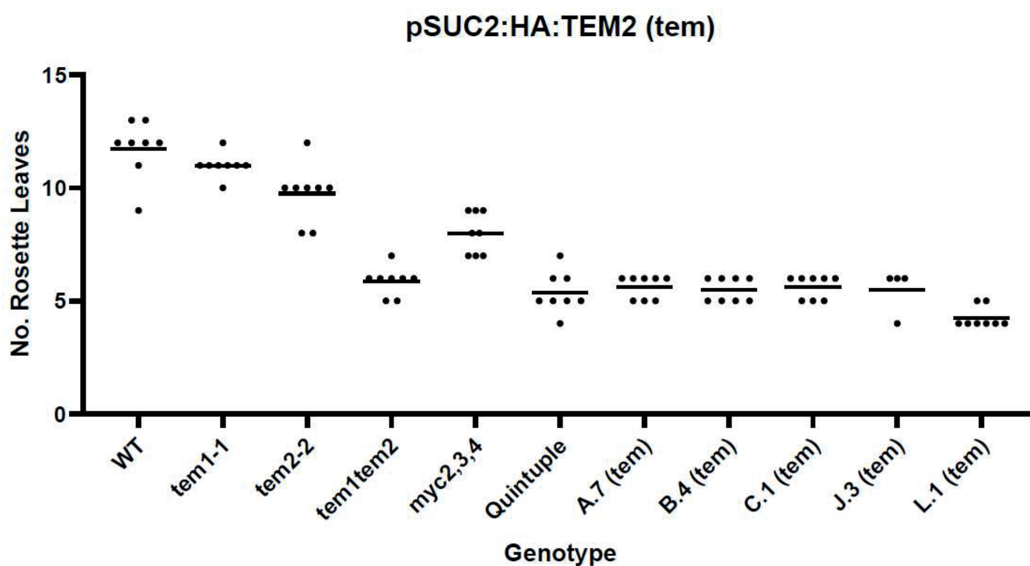
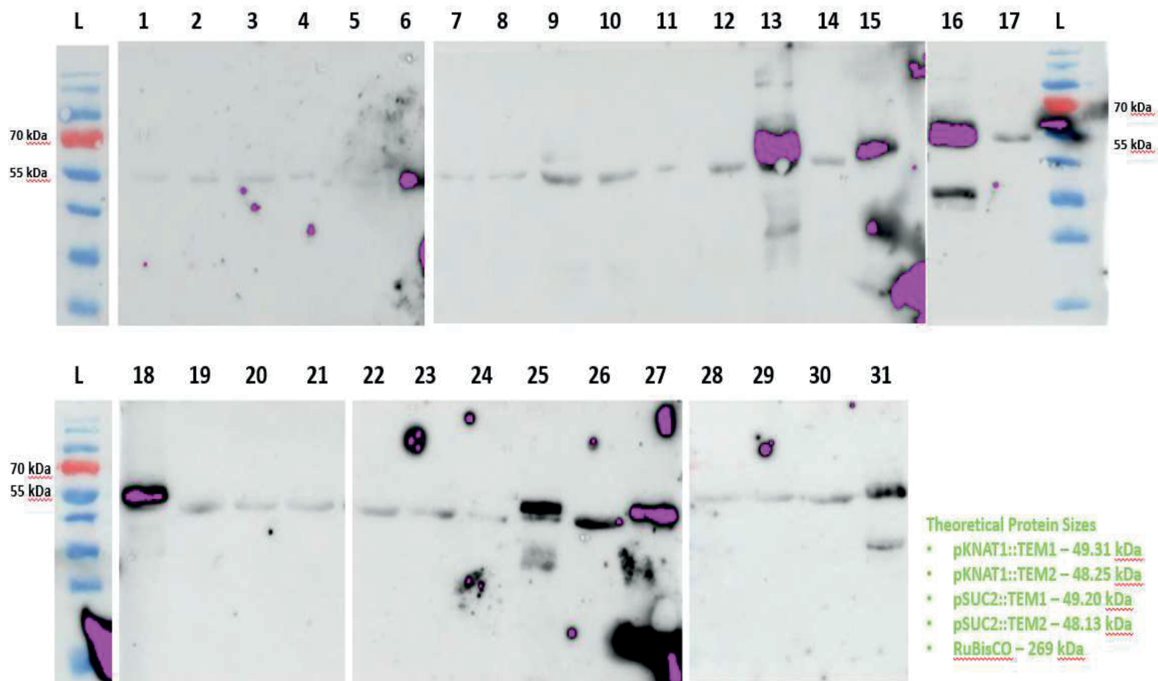


Figure 33: Flowering Time in LD – *tem* pSUC2::HA::TEM2. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

Overall, there was little delay in the mutant and WT transformed plants compared to their respective controls. The differences, if any, were very reduced compared to the untagged construct. The only difference between these experiments was the presence of a 3x-HA tag (necessary to perform ChIP) in the latter experiments. We could only attribute this response to the presence of the tag which could be somehow hindering TEM protein formation or

function. To check if that was the case, we performed Western blots with the more promising lines to estimate TEM protein levels.



No.	Genotype
1	pKNAT1::HA::TEM1 in *tem1tem2* (D.3)
2	pKNAT1::HA::TEM2 in *tem1tem2* (F.4)
3	pSUC2::HA::TEM2 in *tem1tem2* (A.6)
4	pSUC2::HA::TEM2 in *tem1tem2* (B.4)
5	WT (negative control)
6	Positive control for HA::tag
7	pKNAT1::HA::TEM1 in *myc2myc3myc4* (B.2)
8	pKNAT1::HA::TEM1 in *myc2myc3myc4* (D.1)
9	pSUC2::HA::TEM1 in *myc2myc3myc4* (L.1)
10	pKNAT1::HA::TEM1 in WT (B.1)
11	pKNAT1::HA::TEM1 in WT (G.3)
12	pKNAT1::HA::TEM2 in WT (J.1)
13	35S::HA::TEM1 (positive control for TEM1)
14	WT (negative control for TEM)
15	Positive control
16	35S::HA::TEM2 (positive control for TEM2)

No.	Genotype
17	pSUC2::HA::TEM2 in WT (G.2)
18	35S::HA::TEM1
19	WT
20	pKNAT1::HA::TEM2 in *myc2myc3myc4* (D.1)
21	pKNAT1::HA::TEM2 in *myc2myc3myc4* (C.4)
22	pKNAT1::HA::TEM2 in WT (C.1)
23	pSUC2::HA::TEM1 in WT (E.1)
24	pSUC2::HA::TEM2 in WT (G.2)
25	35S::HA::TEM1
26	WT
27	Positive control
28	pSUC2::HA::TEM2 in WT (J.1)
29	pSUC2::HA::TEM1 in *tem1tem2* (Q.2)
30	pSUC2::HA::TEM1 in *tem1tem2* (R.3)
31	35S::HA::TEM2

Figure 34: Western blots showing protein expression of select transgenic lines. Protein extraction of select pKNAT1::HA::TEM1, pKNAT1::HA::TEM2, pSUC2::HA::TEM1, pSUC2::HA::TEM2 (WT, *tem* and *myc*) (chosen on the basis of their flowering phenotype) using 10-day old seedling leaves was done and Western blots performed using anti-HA antibodies. 35S::TEM1, 35S::TEM2 and Col-0 were used as TEM1 positive, TEM2 positive and negative controls respectively.

As figure 34 shows, we did not observe any protein expression in any line. One possibility to explain this could be a rapid protein turnover. Nevertheless, we chose a few lines to perform ChIP experiments on the basis of their phenotypes. We performed qPCRs using primers of known binding regions of TEM target genes. We chose regions of *FT*, *GA3ox1* and *GA3ox2* previously shown to be bound by TEM1.

To be able to compare the binding of TEM in presence or absence of MYC proteins, we used pMYC2::GFP::MYC2 in Col background and crossed it with our *tem* and *myc* mutants. We backcrossed and genotyped the progeny a few times to obtain pure mutant plants with the pMYC2::GFP::MYC2 construct. On phenotyping these plants, we found that they recovered the phenotypes of the *myc* mutant plants close to the WT flowering time (Figure 35). We then used these homozygous lines for the ChIP-qPCR experiments.

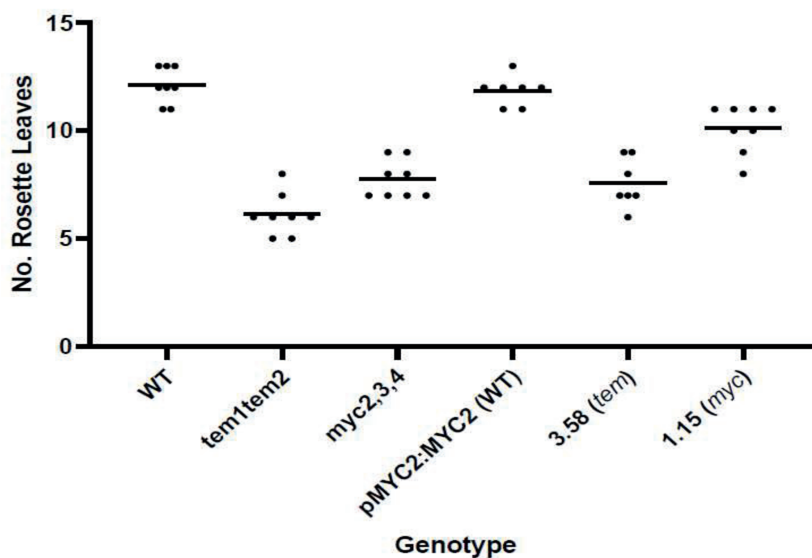


Figure 35: Flowering Time in LD – pMYC2::GFP::MYC2. Rosette leaf number of independent lines compared with WT and mutant plants. Each dot represents number of rosette leaves in individual plants.

The principle behind this experiment was that we would be able to check TEM and MYC binding to target regions in the transformed lines with the help of the tags, HA for the TEM constructs and GFP for the MYC ones. We tested the TEM binding in normal conditions, that is presence of endogenous TEMs and MYCs (in WT), and also in the absence of endogenous TEMs and presence of MYCs (in *tem* mutant) and in the absence of MYCs but presence of endogenous TEMs (*myc* mutant). We also tested MYC binding to the selected

TEM binding regions in normal conditions (in WT), in the presence of endogenous TEMs and absence of endogenous MYCs (in *myc* mutant), and in the absence of endogenous TEMs (in *tem* mutant). Regrettably, we could not test TEM and MYC binding in the absence of both TEM and MYC i.e., in the *myc tem* quintuple mutant.

As we can observe more clearly in the first biological replicate (Figure 36), TEM1, expressed under the control of the *pSUC2* promoter, almost always showed binding to its target regions only in the presence of MYC (in WT and the *tem* mutants). In the absence of MYC (in the *myc* mutant background), binding was only observed in the GA3ox2 promoter region in the second biological replicate (Figure 37). This could mean that TEM1 requires the presence of MYC to be able to bind to its target regions. However, it does not necessarily need to be a direct interaction to MYC, but also an indirect effect. The absence of binding in the *myc* mutant and in the WT in the GA3ox promoter (Figures 36 and 37) could alternatively be explained by competition with the endogenous TEMs.

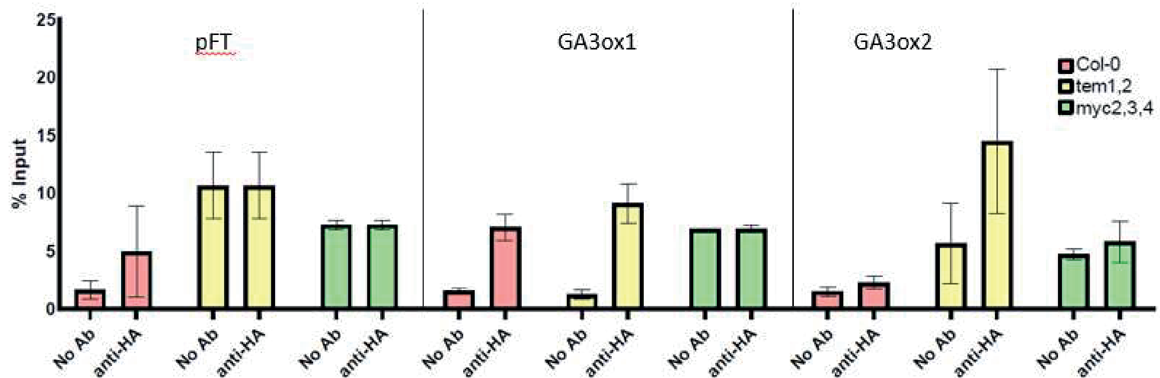


Figure 36: ChIP-qPCR performed using pSUC2::HA::TEM1 transformed lines under LD (Replicate 1). 7-day old seedlings of selected lines were harvested and ChIP performed using anti-HA antibody. qPCRs were done using primers covering proven TEM binding sites on the *FT* promoter, *GA3ox1* and *GA3ox2*. Bars represent % input values of the sample in presence and absence of the anti-HA.

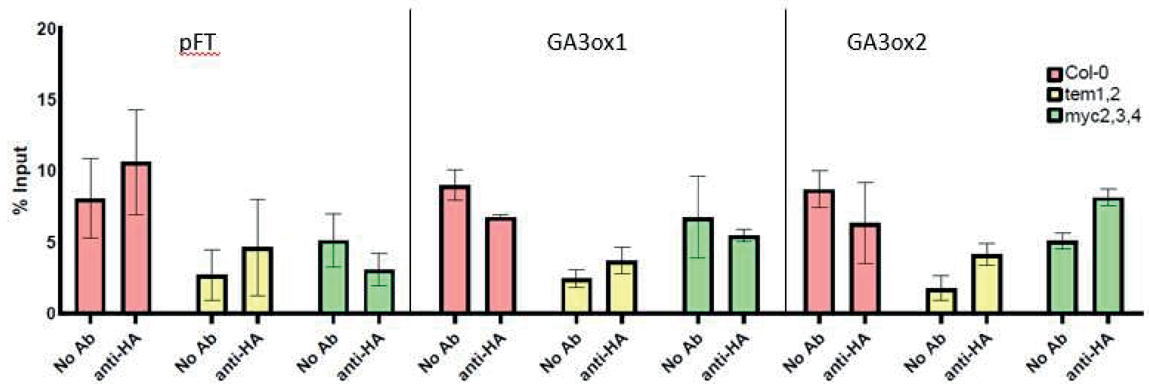


Figure 37: ChIP-qPCR performed using pSUC2::HA::TEM1 transformed lines under LD (Replicate 2)

We then tested the binding of TEM2, controlled by the *SUC2* promoter, to the *FT* and *GA3OX1* and *GA3OX2* regulatory sequences. Similar to TEM1, TEM2 always binds to the *FT* promoter, *GA3ox1* and *GA3ox2* regions in the presence of MYC but no binding is observed in MYC absence (Figure 38 and 39). Except for the *FT* promoter region in replicate 1 (Figure 38), we also detected TEM2 binding to its target sites in the WT background (Figure 38 and 39). In this case TEM2 binding follows the tendency showed by TEM1, indicating that in these circumstances they can act redundantly.

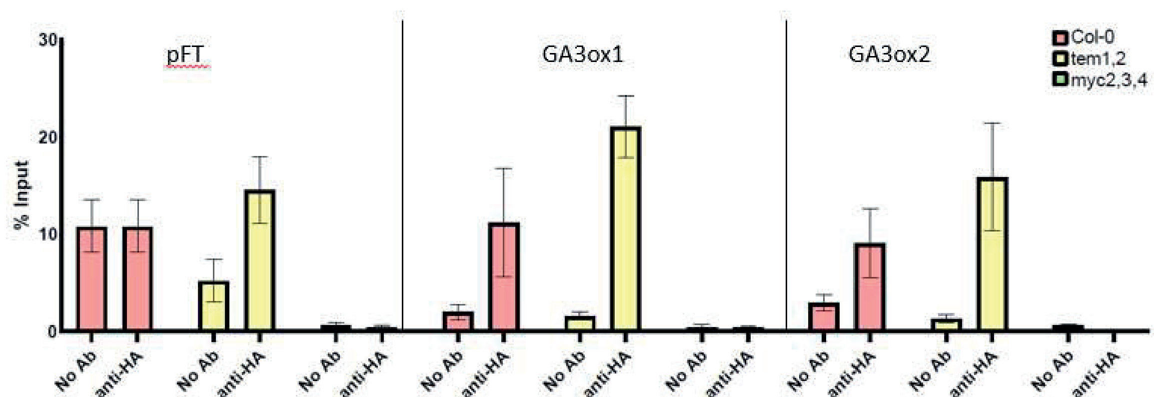


Figure 38: ChIP-qPCR performed using pSUC2::HA::TEM2 transformed lines under LD (Replicate 1). 7-day old seedlings of selected lines were harvested and ChIP performed using anti-HA antibody. qPCRs were done using primers covering proven TEM binding sites on the *FT* promoter, *GA3ox1* and *GA3ox2*. Bars represent % input values of the sample in presence and absence of the anti-HA.

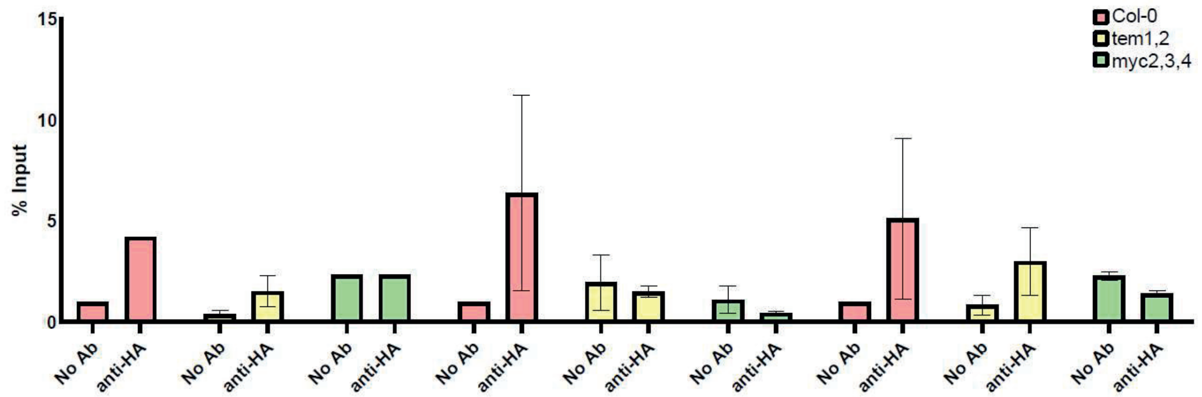


Figure 39: ChIP-qPCR performed using pSUC2::HA::TEM2 transformed lines under LD (Replicate 2)

When we tested the binding of MYC to these same regions, we observed MYC binding to TEM binding sites in the *myc* mutant background (in the presence of TEM) in all the regions (Figure 40 and 41) but we could not detect it in the *GA3ox1* region in the WT background (Figure 40). In addition, we observed some binding in the *tem* mutant background (absence of TEM) in the *pFT* and *GA3ox1* region (Figure 40) but not so in replicate 2 (Figure 41), which may indicate that MYC does not necessarily require the presence of TEM for binding to these genes.

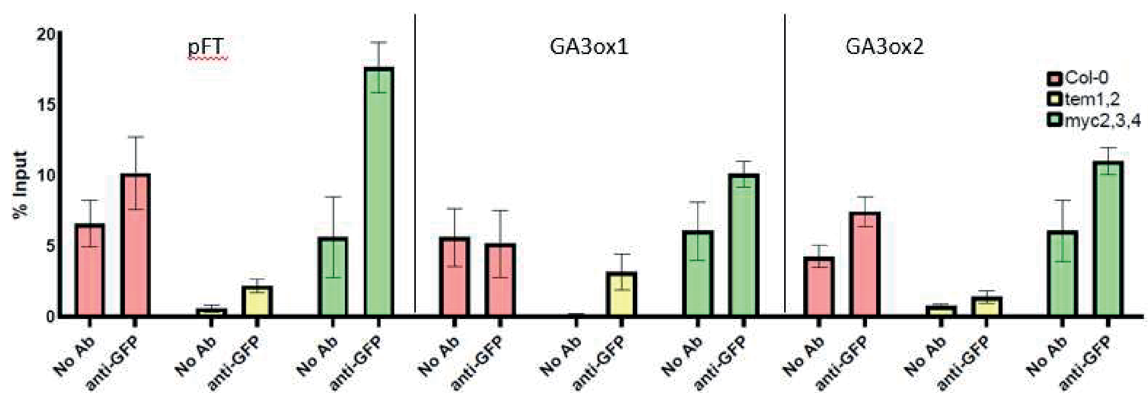


Figure 40: ChIP-qPCR performed using pMYC2::GFP::MYC2 transformed lines under LD (Replicate 1). 7-day old seedlings were harvested and ChIP performed using anti-GFP antibody. qPCRs were done using primers covering proven TEM binding site regions on the *FT* promoter, *GA3ox1* and *GA3ox2* to check MYC binding to the same. Bars represent % input values of the sample in presence and absence of the anti-GFP.

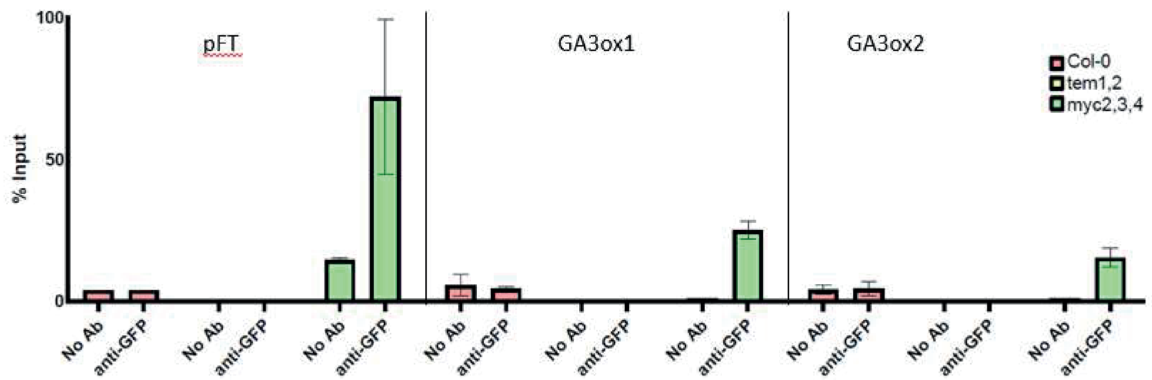


Figure 41: ChIP-qPCR performed using pMYC2::GFP::MYC2 transformed lines under LD (Replicate 2).

As a general conclusion, TEM seems to need MYC for binding to its target *FT* promoter and *GA3OX* sequences, but MYC might not need TEM for binding to the sequences of the three regions we tested.

4.4 Analysing the Effect of Expressing Poplar RAVs in Arabidopsis

The presence of *RAV* genes in non-flowering plants had us curious as to their roles in diverse species. To shed some light on this we looked for *RAV* homolog genes in different species, made constructs under the constitutive promoter 35S, transformed these into *Arabidopsis thaliana*, and studied their effect on flowering times.

We studied the effect of poplar (*Populus trichocarpa*) *TEM* homologs. Upon doing a BLAST, we found 2 predicted *TEM* paralogs in poplar, namely *PtTEM1* and *PtRAV2*. Using these sequences to transform destination vector pAlligator2 and subsequently *Arabidopsis* Col-0, we obtained homozygous transformant lines and performed flowering time experiments on the same. We counted the number of rosette leaves on appearance of the first floral bud, total number of cauline leaves on the plant and the number of days from germination to the appearance of the first floral bud.

As we can see from the following graphs, both TEM1 (Figures 42-44) and RAV2 (Figures 45-47) from poplar delay the flowering times in a Col-0 background, measured by counting rosette leaves (Figures 42 and 45), total leaves (Figures 43 and 46) or number of days (Figures 44 and 47). Although with some variability, all the independent transgenic lines with *PtTEM1* or *PtRAV2* showed a late flowering. 35S::*TEM1* and 35S::*TEM2* plants also show a late floral induction, albeit more drastic. As *Arabidopsis* TEMs, PtTEMs act as floral repressors in *Arabidopsis*. Even though these results are not definitive, they suggest that PtTEMs might have a role to play in poplar flowering as well. Since poplar also has the CO-FT module, poplar TEMs might be instrumental in its regulation, like in *Arabidopsis*.

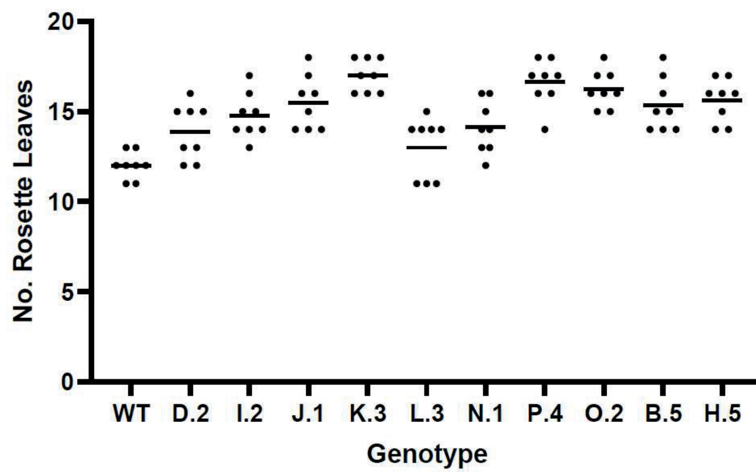


Figure 42: Flowering Time of poplar *TEM1* in LD. No. of Rosette Leaves counted in 35S::*PtTEM1*.

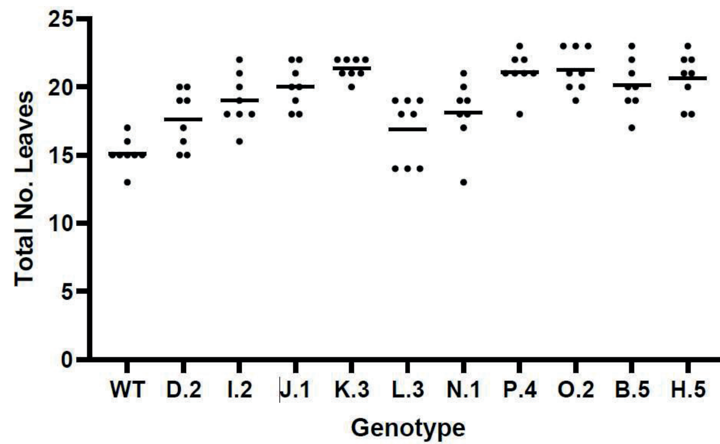


Figure 43: Flowering Time of poplar *TEM1* in LD. Total no. (rosette+cauline) Leaves in counted in 35S::*PtTEM1*.

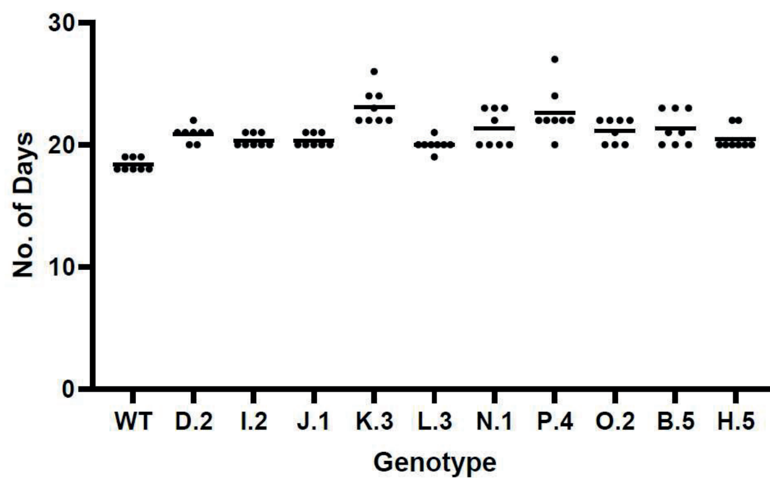


Figure 44: Flowering Time of poplar *TEM1* in LD. No. of days to flowering counted in 35S::*PtTEM1*.

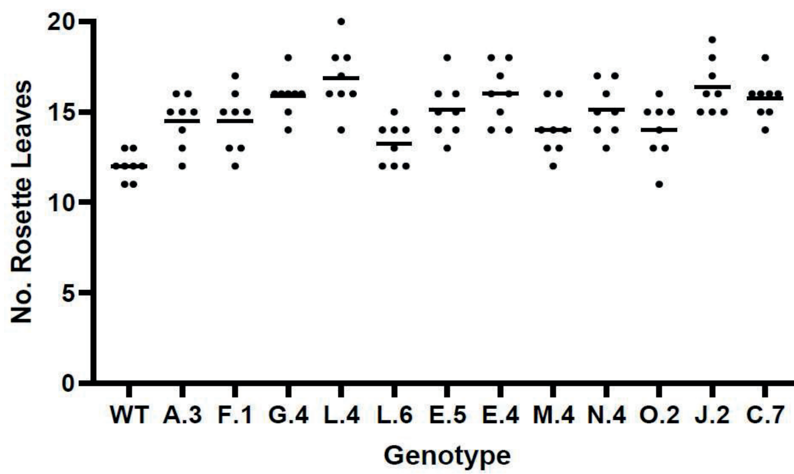


Figure 45: Flowering Time of Poplar *RAV2* in LD. No. of Rosette Leaves counted in 35S::*PtRAV2*.

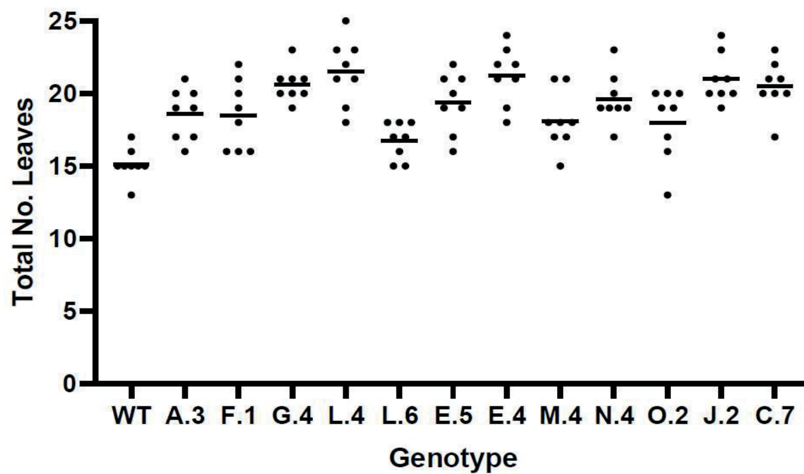


Figure 46: Flowering Time of Poplar *RAV2* in LD. Total No. of leaves counted in 35S::*RAV2*.

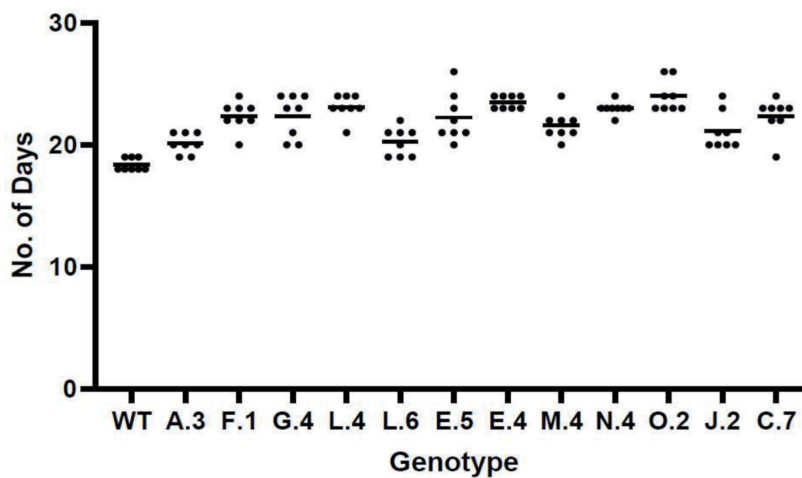


Figure 47: Flowering Time of Poplar *RAV2* in LD. Total No. of days to flowering counted in 35S::*RAV2*

5. DISCUSSION

5.1 TEM and MYC do not regulate *FT* expression by histone modification in the studied regions

TEM and MYC repress flowering by regulating *FT* expression levels through binding to regulatory sequences. It was found that in *tem*, *myc* or *myc tem* mutants the *FT* expression is elevated (Castillejo and Pelaz, 2008, Osnato et al., 2012, Wang et al., 2017, Bao et al., 2019, Osnato and Pelaz, unpublished). However, it is not known how the interaction could affect *FT* regulation, among other targets. In addition, TEM and MYC interact with the corepressor TPL which is involved in modification of histones by interacting with histone modifying enzymes. This, and the fact that TEM and MYC bind to the 5'UTR of the *FT* promoter suggested histone modifications as a possible regulatory mechanism through which they act. Therefore, to study if the early flowering phenotype of *tem* double, *myc* triple and *myc tem* quintuple mutants was a result of differential levels of acetylation or methylation, we did ChIP-qPCR experiments using H3Ac and H3K27me3 antibodies. It was shown that TEMs bind to the 5'UTR of FT, MYC2 in the same region, while MYC3 binds further upstream, ~3kb from the TSS between the CORE 1&2 and E-box elements (Castillejo and Pelaz, 2008, Osnato et al., 2012, Wang et al., 2017, Bao et al., 2019). The complex NF-Y/CO, the main activator of *FT* expression, binds to several regions in the *FT* promoter forming a loop that brings CO to the CORE 1&2 regions, closer to the ATG (Gusmaroli et al., 2002; Wenkel et al, 2006; Ben-Naim et al., 2006; Cai et al., 2007; Kumimoto et al., 2008; Kumimoto et al., 2010; Tiwari et al., 2010; Adrian et al., 2010; Cao et al., 2014; Liu et al., 2014; Siriwardana et al., 2016; Gnesutta el al., 2017a; Gnesutta et al., 2017b). These four established important regulatory regions of the *FT* promoter were analysed, however no significant differences correlating with phenotypes were found in the *tem1tem2, myc2myc3myc4,* or the combination quintuple mutant in either short day or long day growth conditions.

In our experiments, we found that although there were some differences observed in some regions of the *FT* promoter, these were not in tune with the phenotype of the mutants. The results were not consistent among replicates and no conclusions could be drawn from these studies. There are a number of different reasons that could explain this. One reason could be that possible histone changes, if any, are in another regulatory region not studied in our

work. This is corroborated by a recent peer reviewed publication where they did find a significant difference in the histone methylation levels in *tem* mutants, but the changes were found in the coding region downstream of the ATG (Hu et al., 2021). Interestingly they found that TEM1 interacts with CLF (a PRC2 member, which is a known *FT* regulator) in Y2H as well as in co-immunoprecipitation (CoIP) experiments. Their ChIP experiments showed significant differential expression in H3K27me3 levels between WT and *tem* mutants just after the transcription start site (TSS). Although this is not the site where TEM or MYC have been proven to bind, transcription factors have been shown to alter or stimulate expression changes downstream of the site where they bind. It also bears mentioning that they did not find differences in the histone marks upstream in the *FT* promoter (the regions used in our study). Bao et al. (2019) also observed differences in the H3K27me3 levels at a G-box related motif in the *myc3* mutant versus the WT under SD. The TEM/MYC might affect a different area together with TPL or alternatively as a complex they regulate *FT* expression through a different mechanism.

5.2 TEM/MYC seem to regulate *FT* by chromatin remodelling in SD

FT promoter looping is a very important characteristic of the gene which helps activate its expression by facilitating the binding of CO, the main *FT* upregulator. Changes or hinderances to this process could very well be the cause behind many flowering mutants. After testing how compact the chromatin was in *tem*, *myc*, and *myc tem* mutants compared to wild type plants under LD, using FAIRE to measure the nucleosome occupancy, we found no remarkable differences in the tested genotypes, despite having characteristically distinct phenotypes that could have been explained by having a much more accessible chromatin. The reason for this lack of differences could be that under LD, the chromatin has reached its utmost accessibility limit and can therefore not be freer. Another possible reason could be that by the time sampling of tissue was done (7-day old seedlings at ZT16), chromatin changes had already occurred and therefore could not be observed in these experimental conditions, and collecting tissue at an earlier age might show differences. Yet another possibility could be that chromatin changes occurred at a site other than the ones we studied and therefore we could not observe them. Another factor could also be contributing to the phenotype changes observed in these genotypes.

Under SD, however, the quintuple mutant had a higher ratio of free versus bound DNA compared to the rest of the samples in all regions tested. This suggests that flowering time may be repressed by TEM and MYC through chromatin remodelling of the *FT* promoter. Even though *FT* is not a major causal factor in determining flowering time under SD in WT plants because of the low CO availability, the mutant genotypes of *tem*, *myc* and *myc tem* have much higher levels of *FT* compared to the controls even under SD (Osnato and Pelaz, unpublished; Bao et al., 2019). In this scenario of open chromatin under SD, the activator CO, despite being present in low levels, can reach its target sequences for *FT* activation. Transcription factors being unable to reach their target binding site is one of the major ways to regulate gene expression, therefore it is reasonable to say that differences in nucleosome occupancy in the flowering time mutants could be responsible for their corresponding phenotypes.

It has been reported that MYC2, MYC3 and MYC4 bind to G-box or its related motifs (Fernández-Calvo et al., 2011). In *FT* as well, MYC2 binds to two G-box related elements, one just near the TSS close to where TEM binds, and the other further downstream in the intronic region of *FT* (Wang et al., 2017).

Under SD, MYC3 has been proven to play a major role in floral regulation by regulating *FT*. In *myc3* mutants, under SD, *FT* has been shown to be upregulated and have a similar diurnal expression pattern to LD, with a peak at ZT16. However, this phenomenon was not observed under LD because the higher amounts of GA would release MYC3 repression. This proved that MYC3 is a flowering repressor only under SD, whose repressive action is stabilised by DELLA proteins. In addition, ChIP assays showed MYC3 competing with CO to bind directly to a ACGGAT motif of the *FT* promoter which is ~3kb upstream of the ATG and a region involved in *FT* looping and its subsequent activation. Chromatin conformation capture (3C) assays also showed differences in interaction at the same locus in the *FT* promoter between the *myc3* mutant and the WT (Bao et al., 2019). This means that *FT* repression by MYC3 under SD occurs because of direct binding of MYC3 to the *FT* promoter and affecting the CO-FT loop formation.

Binding sites of both MYC2 and MYC3 to G-box related motifs and MYC3 action of *FT* under SD support our results completely and could very well be the reason behind the differences in nucleosome occupancy detected in our experiments.

MYC could then repress *FT* expression via two different mechanisms under SD; MYC3 would bind a region 3kb upstream of the ATG affecting the *FT* looping and establishing H3K27me3 repressive mark, and MYC2/TEM would regulate chromatin remodelling compacting the *FT* promoter.

5.3 TEM seems to require MYC for DNA binding

Both TEMs and MYCs have been shown to regulate flowering in *Arabidopsis* by binding to *FT*. They then have at least one common target, but the quintuple mutant phenotype is stronger than that of the *tem* and *myc* mutants, which suggests that they may also have independent targets.

In order to be able to perform ChIP experiments to test whether TEM and MYC require each other for binding, antibodies against these proteins are required. However, because of the lack of specific antibodies against TEM and MYC, tagged versions against commercial antibodies were used. For TEM we generated a chimera protein fused to the human influenza hemagglutinin (HA) tag to use commercial anti-HA antibodies. This chimera was then placed under the control of the *TEM1* promoter resulting in the pTEM1::HA::TEM1 construct. When testing the functionality of this construct, we found that HA::TEM1 expressed under the control of pTEM1 in *tem* mutant background could not recover the phenotype of the mutant phenotype, and it did not delay the WT flowering time either when introduced in a Col-0 background. We hypothesized that the sequence used as TEM1 promoter was not enough for the correct expression of *TEM1* and some regulatory sequences were missing. To overcome this problem we used other promoters that induce tissue specific expressions in shoot apical meristem and in the leaf vascular tissue, *pKNAT1* and *pSUC2* respectively, and fused them to *TEM1* and *TEM2* cDNA sequences resulting in pKNAT1::HA::TEM1, pKNAT1::HA::TEM2, pSUC2::HA::TEM1 and pSUC2::HA::TEM2. Surprisingly, these tagged constructs did not recover the mutant phenotype up to the same levels as the untagged construct making us wonder if the 3xHA tag was somehow interfering with the proper gene expression and/or protein function. Perhaps the protein conformation changed resulting in it becoming non-functional or it promoted a rapid degradation, supported by the fact that we could not detect the chimeric proteins in western blots.

Because few lines showed a very weak effect, we decided to proceed using some of the pSUC2 lines as they showed the "best" phenotype recovery to perform the ChIP experiments. We then used pSUC2::HA::TEM1, pSUC2::HA::TEM2 and pMYC2::MYC2::GFP (Lorenzo et al., 2004) lines to test their requirement for the other to bind to different regions of *FT, GA3ox1 and GA3ox2* sites (proven TEM targets).

Considering both replicates, we observed binding of TEM1 or TEM2 to their target sites only in the presence of MYC (*tem* mutant and/or WT background). This could mean that TEMs need MYCs to bind to their target sequences but this necessity might not necessarily be direct. Although, the fact that TEMs and MYCs interact in Y2H and Bi-FC experiments suggest a direct requirement, their interactions could occur through another TF or formation of a bigger complex with both of them. On the contrary, when tested MYC2 binding to the TEM target genes, we observed binding to the three tested genes in the presence of TEM (*myc* mutant) but also in its absence (*tem* mutants and WT) in the *FT* and *GA3OX1* genes. This might mean that for binding to *FT* and *GA3OX1* sequences at least, MYC2 does not seem to require the presence of TEMs. TEMs may only be needed to bind to *GA3OX2*. Thus, these results suggested that MYC2 binds to TEM target regions although this could be through a direct contact of MYC2 to these sequences or alternatively through indirect binding through TEM, which is a proven regulator at these loci. In the second case, the interaction with MYC might help further repression of *FT* and *GA3OX1* genes. While these results are not conclusive by any means, they suggest that perhaps there is a direct or indirect interaction between these transcription factors which affects their binding to target genes. This interaction might even be required for their optimal repression of flowering.

It has been shown that in the absence of MYCs or TEMs *FT* expression is upregulated (Castillejo and Pelaz, 2008, Osnato et al., 2012, Wang et al., 2017, Bao et al., 2019). Furthermore, in our laboratory it was observed that the upregulation of *FT* is higher in the quintuple *myc tem* mutants relative to the WT, however, *GA3OX1* is only upregulated in the absence of *tem* (Osnato and Pelaz, unpublished). Our results suggest that TEM probably requires MYC for DNA binding, but MYC does not need TEM, and the absence of one of them would affect their function because transcription regulation of their targets is affected when only one of the partners, TEM or MYC, is absent. In *tem* mutans, even if MYCs are present and able to bind, there is upregulation of *FT* and *GA3OX1* genes. In *myc* mutants, TEM might not able to bind to its target genes, and should show similar upregulation and

therefore phenotype as in *myc tem* mutants. However, *FT* expression is further upregulated in quintuple mutants, which flowers earlier, indicating that some function and binding might remain when one of the partners is missing. The regulation of *GA3OX1* seems more difficult to convene, it is upregulated in *tem* mutants (when only MYCs are present) similarly to *myc tem* (neither TEMs nor MYCs are present), but it is not affected in *myc* mutants (only TEMs present) and similar to Col (both TEMs and MYCs present). This would indicate that MYC, even if bound to the *GA3OX1* region, would require TEM for its function, however TEMs were able to fully repress *GA3OX1* without the capability to bind to DNA in the absence of MYCs, which then might suggest that TEM may act through interaction with other binding proteins. The fact that *myc tem* mutants show an earlier phenotype than *tem* and *myc* mutants also indicates that MYC and TEM independently retain some flowering repressive capabilities.

5.4 Poplar TEM homologs repress flowering in *Arabidopsis*

Even though RAV homologues have a diverse array of functions in different species, their role in somehow controlling flowering time seems to be conserved in many of them. The RAV family of TFs plays an important role in various physiological processes during normal growth and stress conditions. Their presence in all land plants with the two DNA binding domains mostly conserved speaks to its significance in regulating fundamental functions. Although, many roles of members of this family have been uncovered, it has not been studied much in poplar.

Two FT like genes, *PtFT1* and *PtF2*, have been identified in poplar (Böhlenius et al., 2006; Hsu et al., 2006). They are expressed in different tissues and in different seasons, the former in the stem and apical buds in late winter and the latter in the leaves in early summer. Their constitutive expression caused a late flowering phenotype in transgenic poplars (Böhlenius et al., 2006; Hsu et al., 2006). *PtFT2* suppression in autumn mediated a growth cessation response, highlighting their role in dormancy (Hsu et al., 2011). 14 *PtCOL* (*CO-like*) are also present in poplar. They have a conserved B-box and CCT domain and show diurnal oscillation, suggesting their involvement in photoperiod responses (Li et al., 2020). Besides this, over-expression of *CsRAV1* (*Arabidopsis TEM* homolog) in poplar hybrids induced sylleptic branching although their RNAi lines did not affect growth. Even though the exact

functions of the CO-FT model in poplar have not yet been uncovered, the conservation of these genes might mean similar underlying mechanisms.

In this study, we identified two *TEM* homologs and expressed them in *Arabidopsis* using the constitute 35S CaMV promoter. The transgenic *Arabidopsis* lines showed a late flowering phenotype with respect to the number of rosette leaves, total number of leaves and number of days compared to the control, Col-0. This phenotype is consistent with the 35S::TEM1 and 35S::TEM2 phenotypes, although much less severe. Even though the experiment is not done in the native host, it does give a hint as to the possible role of poplar TEMs' action in Arabidopsis.

Therefore, the constitutive expression of poplar *TEM* homologs in *Arabidopsis* showed a consistent late flowering phenotype in different lines, hinting at them at least being capable of utilizing the same machinery in *Arabidopsis* flowering network and being able to influence it. This is plausible because poplar has similar molecular modules including the presence of *GI*, *CO* and *FT* orthologs, which although present in different regions and control different processes share similarities with the Arabidopsis one.

6. CONCLUSIONS

- Differences in flowering times of WT, double *tem1 tem2,* triple *myc2 myc3 myc4* and *tem1 tem2 myc2 myc3 myc4* quintuple mutants in LD or SD were not due to H3 acetylation or H3 lysine trimethylation on the four established *FT* promoter regulatory regions.
- The repressive action of TEM or MYC is not caused by modification of histone marks on the four *FT* promoter regulatory regions.
- Chromatin remodelling on the *FT* promoter is not responsible for the differences in flowering times of WT, *tem1 tem2, myc2 myc3 myc4* and *tem1 tem2 myc2 myc3 myc4* mutants in LD.
- Differences in the nucleosome occupancies on the *FT* promoter regions might be responsible for the early flowering phenotype of the *tem1 tem2 myc2 myc3 myc4* mutants in SD.
- TEM1 and TEM2 bind to their target loci, *FT* promoter, *GA3ox1* and *GA3ox2*, more prominently in the presence of MYC.
- MYC binding to TEM target loci does not necessarily depend on the presence of TEM.
- *Populus trichocarpa TEM* (*PtTEM*), homologs, *PtTEM1* and *PtRAV2*, act as floral repressors when expressed in *Arabidopsis*.

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