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Flowering time regulatory network controlled by FLOWERING LOCUS C (FLC) in response to temperature changes

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ABSTRACT

The global temperature has been fluctuating drastically due to global warming. This has major effects on living organisms around the world, especially in sessile organism such as plants. In flowering plants, initiation of flowering is a crucial developmental event, which requires both internal and environmental signals to determine when floral transition should occur, in order to maximize reproductive success. One of the key environmental signals that determine flowering time is ambient temperature. In this study, we aimed to investigate the influence on flowering of daily variable temperature, where temperature fluctuates throughout the day, in selected *Arabidopsis* accessions with different alleles of the key flowering regulators, FLOWERING LOCUS C (FLC) and FRIGIDA (FRI), namely Col-*0*, C24 and their late flowering hybrid, C24xCol-*0*. Our result showed that Col-*0*, C24 and C24xCol-*0* grown in a constant ambient temperature flowered later than those grown in a variable temperature mimicking daily fluctuation in both wild-types and late

flowering hybrid. Besides, we have explored the connection between FLC and its up- and downstream regulatory genes, in response to temperature changes, using publicly available data. The transcriptional changes of flowering genes were observed as a result of a temperature shift, which were combined with an FLC- associated flowering time regulatory network to reveal the transcriptional dynamics in temperature shift conditions. The network can be further expanded to cover other flowering genes and thus provide more comprehensive model of expression dynamics of flowering genes in response to temperature changes.

Keywords: Variable temperature, FLC, Flowering, *Arabidopsis thaliana*

INTRODUCTION

Initiation of flowering is one of the important developmental stages in higher plants. To maximize their reproductive success, plants need to flower at suitable time, and this decision is highly dependent on several internal signals and environmental factors. Temperature is known to be one of the most essential cues that regulate flowing time. For instance, certain plants require prolonged exposure of cold, known as vernalization, to trigger flowering. In the model plant *Arabidopsis thaliana,* accessions are generally classified into the winter-annual and summer-annual groups. The winter-annual accessions exhibit a late-flowering phenotype that requires vernalization, while the summer-annual accessions exhibit an early-flowering phenotype, and flower rapidly without the requirement of vernalization. The difference in flowering behaviors are partly due to the allelic variation of two loci: FLOWERING LOCUS C (FLC), which is one of the key flowering repressors, whose activity can be suppressed by vernalization (Koornneef et al. 1994; Lee et al. 1994) and/or reducing the activity of its upstream activator FRIGIDA (FRI) (Napp-Zinn 1979; Lee, Bleecker, and Amasino 1993; Clarke and Dean 1994). In addition to vernalization response, ambient temperature is one of the key environmental stimuli that highly influences flowering time, especially when temperature has been increasing due to the global warming. Earlier studies have showed that high ambient temperature can accelerate transition to flowering, whereas low temperature leads to delayed flowering

(Balasubramanian et al. 2006; Samach and Wigge 2005; Blazquez, Ahn, and Weigel 2003). However, the majority of studies focused on constant temperature, which might not represent the temperature conditions in natural environments. Interestingly, Burghardt et al. (2016) have showed that daily variable temperature, where temperature fluctuates during the day, can mitigate the effect of flowering repressors, leading to earlier flowering (Burghardt et al. 2016). However, the molecular mechanism of this process is only partly explored. In our study, we aimed to investigate the effect of daily variable temperature in different *Arabidopsis* accessions with different alleles of the key flowering regulators, FLC and FRI, namely Col-*0*, C24 and their late flowering hybrid, C24xCol-*0*. Moreover, we have explored the connection between FLC and its up- and downstream regulatory genes, in response to temperature changes, using publicly available data and bioinformatic tools. This study contributes to a better understanding of how plants adapt to new environments, in terms of flowering, especially in the light of global warming, and may pave the way to increase crop production in the future.

MATERIAL AND METHODS

Plant materials and growth conditions

The seeds of *Arabidopsis thaliana* accessions Col-*0*, C24 and C24xCol-*0* were stratified in 0.1% ½ MS (Murashige and Skoog 1962) agar solution and kept in the dark at 4°C for 3 days. After stratification, the seeds were sown in compost (Levington's F2) and grown in growth chambers (MLR 351, SANYO Electric Co., Ltd.) under cool-white fluorescent light in the short-day (SD) condition (8 h light/16 h dark), at either constant temperature, "CON" (22°C), or variable temperature, "VAR" (average around 22°C). The variable temperature profile was obtained from the study by Burghardt et al. (2016).

Flowering time analysis

The flowering time of Col-*0*, C24 and C24xCol-*0* grown under CON and VAR conditions was measured as the total number of days to flowering (when the inflorescence was about 1 cm in length). The total number of days were displayed as jitter plots using the "ggplot2" package in R (https://www.r-project.org). P-values between CON and VAR conditions were computed by Wilcoxon rank sum test using the stat_compare_means() function in the "ggpubr" package.

Differential gene expression and network analyses

To investigate the transcriptional dynamics of FLC, which is one of the potent floral repressors, and its up- and downstream genes at different temperature conditions, a list of flowering genes were collected from the Flowering Interactive Database (FLOR-ID) (Bouche et al. 2016), and transcripts per million (TPM) values of those genes were extracted from publicly available RNA-seq data of *Arabidopsis* Col-*0* grown under different temperature conditions in previous study by Pajoro et al. (2017) (GEO accession GSE85282). Z-scores for each gene were calculated across the temperature conditions using the "matrixStats" package in R, and were visualized as heatmap using the "ComplexHeatmap" package. To further explore the interplay between FLC and its up- and downstream genes in response to temperature changes, TPM values were used to compute the correlation of expression patterns between genes pairs under temperature shift conditions using Spearman's rank correlation coefficient method. The correlation coefficients were computed using the cor() function and P-values of the correlation were determined using the cor_pmat() function in the "ggcorrplot" package in R. We then integrated gene regulatory information and correlation coefficient values of each gene pair to construct a co-expression - gene regulatory network using Cytoscape (www.cytoscape.org).

RESULTS AND DISCUSSION

Daily variable temperature can accelerate flowering in late-flowering plants with functional FLC and FRI

Sanda and Amasino (1995) previously showed that the F1 plants of the crosses between C24 and Col-*0* contain functional strong alleles of FRI and FLC from C24 and Col-*0*, respectively, resulting in extremely late flowering compared to their parents under constant temperature condition (Sanda and Amasino 1995). In our work, we have studied the influence of daily variable temperature on *Arabidopsis thaliana* wild-types Col-*0*, C24 and their late flowering hybrids (C24xCol-*0*). Plants were grown at either constant temperature of 22°C (CON) or variable temperature (VAR), which temperatures range from 12°C to 32°C, under the short-day (SD) condition (Figure 1A). Phenotypic differences including early flowering, leaf shape and petiole elongation were observed in the VAR condition (Figure 1B). Consistent with the previous study, our results showed that in CON, C24xCol-*0* exhibited late flowering relative to both parents (Sanda and Amasino 1995). Interestingly, both wild-types and their hybrids grown in VAR flowered earlier than those grown in CON. This suggested that variable temperature can accelerate flowering in late flowering hybrid with functional FLC and FRI. Moreover, the difference in flowering time between CON and VAR was more noticeable in C24 than Col-*0,* indicating that this response might be accession-specific (Figure 1C).

Transcriptional dynamics in response to temperature changes

To explore the transcriptional patterns of up- and downstream of FLC that might play a role in the response to ambient temperature changes, we extracted the transcription levels of these FLC-associated genes from the RNA-seq experiments in shoot apical meristem (SAM) tissue of *Arabidopsis thaliana* accession Col-*0* from an earlier study by Pajoro and colleagues (Pajoro et al. 2017). In their experiments, plants were grown in two independent temperature shift conditions under the short-day photoperiod (8 h light/16 h dark). In the first experiment, plants were grown at constant 16°C for 5 weeks and then either kept at 16°C, or transferred to 25°C for 1 day, and SAM tissue samples were harvested between ZT4 and ZT7. In the second experiment, plants were grown at 16°C for 5 weeks and then either kept at 16°C, or transferred to 25°C for 1, 3 and 5 day(s) and tissue samples were harvested at ZT4. Differentially expressed genes under different temperature shift conditions are shown in Figure 2. We observed unique expression patterns between the two types of experiments (indicated by the purple and yellow bars at the top of heatmap), suggesting that the distinct patterns might be specific to the time of day of sample collections (ZT4 vs' ZT4-7). However, in the temperature shift samples collected at ZT4 (yellow bar at the top), we observed that the genes in Cluster C (*e.g.* SVP, FCA) were up-regulated within 1 day after plants were moved to 25°C, indicating that those genes rapidly response to the temperature change, whereas Cluster D (*e.g.* SPT16, SPL15, GASA5) displayed slower up-regulation at 3 days after the shift. In addition, most of the genes in Cluster B (*e.g.* FRI, ICE1) were transiently down-regulated at day 1 after temperature shift, and returned to the original levels within 3 days. These results showed that the flowering genes, at least for those directly associated with FLC, have different unique transcriptional dynamic patterns, when they are subjected to temperature changes.

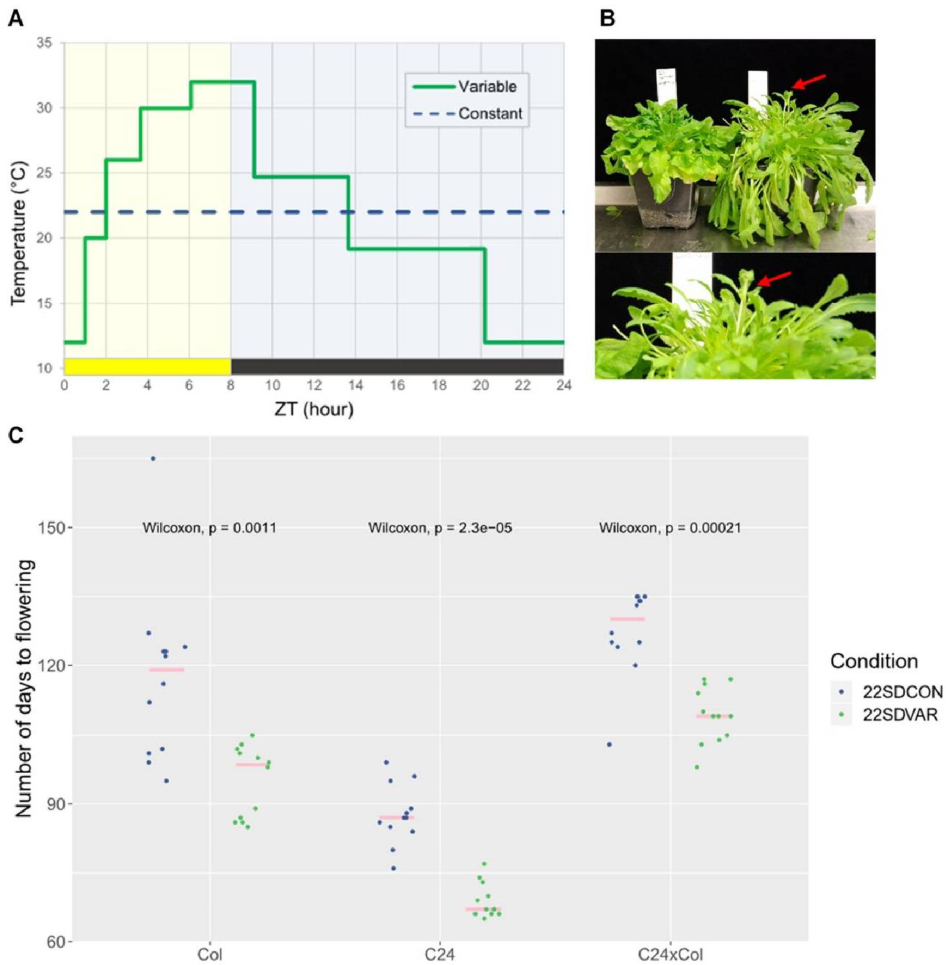

Figure 1. Phenotypic changes including early flowering, petiole elongation in response to daily variable temperature. (A) The profiles of constant (CON) and variable (VAR) temperature conditions under the short-day (SD) photoperiod used in this study (adapted from Burghardt et al., 2014) The rectangles below the graph describe light conditions under

short-day cycle (8 h light/16 h dark). ZT, Zeitgeber time. (B) C24xCol-*0* grown in CON (left, upper panel) and VAR (right, upper panel) conditions at 115 days after sowing (DAS). First flower bud of C24xCol-*0* grown in VAR (lower panel). (C) Jitter plot showing the total number of days to flowering of Col-*0*, C24 and C24xCol-*0* grown under the CON (blue) and VAR (green) conditions. Pink lines represent medians. Total number of plants in each line of *Arabidopsis thaliana* = 12. p represents P-value, computed by Wilcoxon rank sum test.

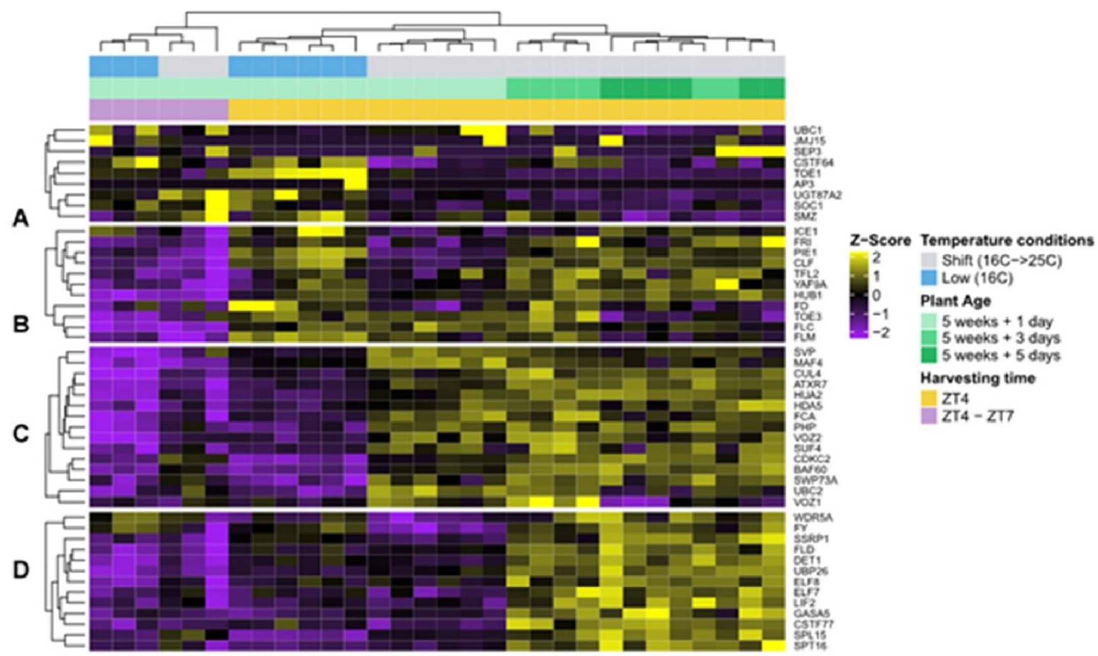

Figure 2. The transcriptional dynamics of FLC and its up- and downstream genes in response to temperature shift. Heatmap showing the transcriptional patterns of FLC and 44 up- and downstream genes in shoot apical meristem (SAM) of *Arabidopsis* Col-*0* grown under temperature shift conditions (from 16°C to 25°C). The genes were hierarchically clustered into four groups (A, B, C and D). Yellow indicates relatively up-regulated genes and purple indicates down-regulated genes.

The co-expression – transcriptional regulatory network model of FLC-mediated flowering time control

To further investigate the interplay between FLC and its up- and downstream genes and the influence of temperature changes, we integrated the correlation coefficient values of transcriptional patterns of each gene pairs, and the gene regulatory information (*i.e.* activator, repressor), which were obtained from the Flowering Interactive Database (FLOR-ID) (Bouche et al. 2016) and published articles, to construct a combined co-expression – transcriptional regulatory network, as shown in Figure 3.

In general, one may expect to see the correspondence between expression correlation values and the types of regulation, for example, the transcriptional activators should tend to be co-expressed or correlated with their target genes, and the repressing genes would be inversely correlated. In fact, we observed that this was true for some genes in the network but not all, as described in more details below. In our network, we could cluster the FLC-associated genes into three groups. Group A is consisted of the genes whose expression correlations agree with the regulatory types (*i.e.* activating or repressing) reported in previous studies, and some of them showed the strong evidence of the connection between two genes. For example, we observed that the expression of FLC, which is known as a key flowering repressor, was highly negatively correlated with SPL15. Consistent with our finding, it has been reported that FLC directly binds to SPL15 promoter, and the SPL15 mRNA level increased in the *flc* mutant, indicating that FLC acts as direct upstream repressor of SPL15 and represses its expression (Deng et al. 2011). Other genes in Group A include FCA, CDKC2 and HDA5.

On the contrary, the expression correlations of the regulators and targets in Group B appeared to be the opposite of what had been found by other studies. A possible explanation is that, those upstream regulators might play an indirect role in regulating the FLC expression, possibly interacting with other proteins, and the exact regulatory mechanisms remain unclear. For example, our result showed that the FLC expression appeared to be highly negatively correlated with GASA5 and SPT16. However, previous studies showed that the FLC transcription level decreased in both *gasa5* and *spt16* mutant lines, suggesting that GASA5 and SPT16 may up-regulate FLC. However, the exact regulatory mechanism has not been intensively investigated and it is not yet clear whether those upstream regulators regulate the expression of FLC by binding to FLC directly or through other proteins (Zhang et al. 2009; Lolas et al. 2010). Moreover, since the expression correlation was computed from the shoot apical meristem dataset, it should be considered that the regulatory mechanisms and gene expression patterns might differ in different tissue types. Other genes in Group B include FRI, ELF7 and ELF8.

In Group C, we found that the FLC-associated genes not only interact with FLC but also with other regulators, especially two main floral integrator genes including SOC1 and FT. These genes act as downstream of many regulators to integrate the signals from multiple flowering pathways. We observed that the expression of floral repressor FLC was negatively correlated with FD, which forms the protein complex with floral integrator, FT and this FT-FD complex are required for induction of the SOC1 expression at the shoot apical meristem, to promote transition to flowering as shown before by other studies and it was reported that FLC might be a direct transcriptional repressor of FD (Searle et al. 2006; Abe et al. 2005). Moreover, we also found that the expression of FD was positively correlated with SOC1, falling in line with FD being one of the activator of SOC1.

We note that, even though some genes are known to be associated with FLC and to one another, the information on the regulatory type (*i.e.* activator or repressor) might not be available due to the lack of direct experimental evidence, such as loss-of-function mutation from published articles. For instance, SEP3 appears to be upstream regulator of FD, and we have showed that their expression patterns are negatively correlated. This might suggest that SEP3 might be a repressor of FD, at least in SAM and in the context of this temperature shift. We foresee that this network model might help predict the functions and the types of regulation of uncharacterized genes in similar manner.

To improve the coverage and accuracy of this network model, we aim to increase the number of datasets from RNA-seq experiments, which were performed under different temperature conditions, to calculate the expression correlation, and reconstruct the network that contain more flowering genes.

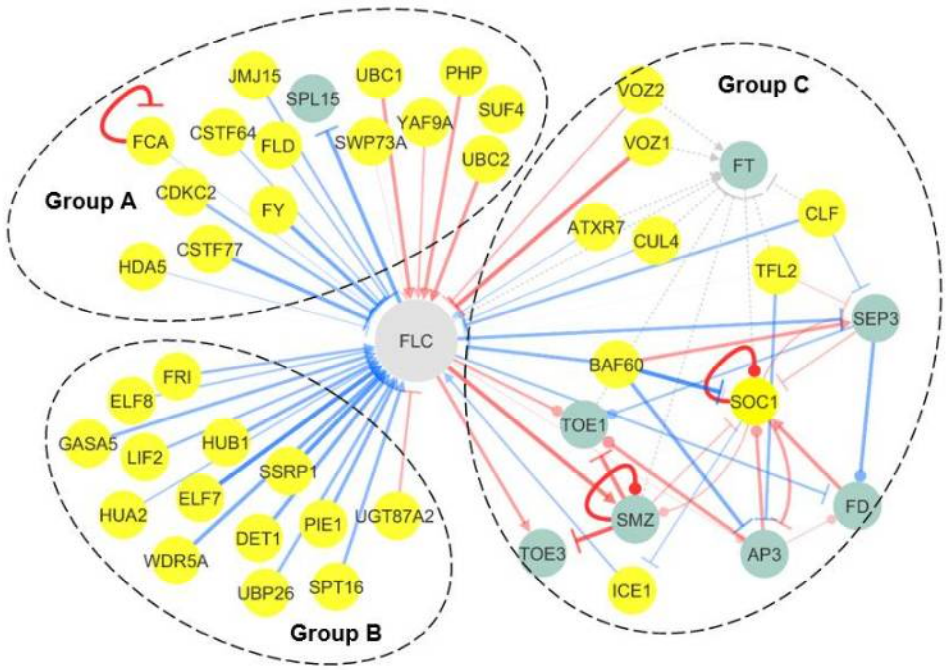

Figure 3. The network model partially explains the FLC-mediated regulation of flowering time. Node colors represent upstream (yellow) and downstream (green) actors of FLC. Edge colors represent correlation coefficient including positive (red) and negative correlation (blue), using Spearman's correlation coefficient method. Solid lines with arrow, block and circle represent activation, repression and uncharacterized regulation, respectively. Low correlation values are shown as transparent/paler edges and high correlation values as darker edges. Thicker edges correspond to lower P-values.

CONCLUSION

Here we have demonstrated that, not only the elevated temperature, but fluctuations in temperature during the day also accelerates the floral transition. This study also demonstrates a conceptual model of gene regulatory mechanisms in controlling flowering time under temperature changes. By combining transcriptional regulatory information with expression patterns from transcriptomic studies, we showcased how a network model could be used to characterize the functions and the types of regulation of FLC and its neighboring genes in the context of flowering time control. We expect that, this study may provide better understanding of how plants perceive and respond to environmental conditions such as daily temperature fluctuations, by focusing on flowering time, and provide a platform to breed plants that can adapt to new environments, may pave the way to increase crop production in these environments.

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