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Post-transcriptional regulation of the expression of the flowering time gene FT in different light conditions

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The shift from a vegetative to a reproductive phase is orchestrated by a number of genes including *CONSTANS (CO), FLOWERING LOCUS T (FT),* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1).* Many plants, both perennial and annuals, including *A. thaliana,* initiate this transition in response to changes in day length. The light signal is perceived in the leaves and transmitted to the apex where it induces flowering and the *FT* mRNA has been found to be part of this signal. In order to study the regulation of *FT,* a heat inducible system has been used in this work. In this report I confirm that the flower initiation caused by activation of a *Hsp::FT Hsp::GUS* transgene requires light. Heat shock induction of *GUS* and *FT* suggests that there is no difference in the induction kinetics or the relative induction levels between the two constructs in light and dark, suggesting that the light conditions are not affecting the transcriptional regulation. Instead, my data suggest that *FT* expression in different light conditions might be controlled by a post-transcriptional regulation possibly including both *FT* mRNA stability and the efficiency of translation or stability of the FT protein. This regulation might contribute to the reduced efficiency of FT-induced flowering in darkness.

INTRODUCTION

The initiation of flowering in *Arabidopsis thaliana* is mediated by four pathways, these are referred to as the photoperiod response-, autonomous-, vernalization-, and gibberellin pathways. This crucial moment in the development of the plant, the shift from a vegetative to a reproductive phase, is orchestrated by a number of genes including *FLOWERING LOCUS T (FT)*, and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* as the pathways converges into an integrating pathway (Komeda, 2004). Many plants, including *A. thaliana*, initiate this transition in response to day length. Plants grown in long day conditions (LD, 16 h light) will start to flower earlier than plants grown in short day conditions (SD, 8 h light) (Searle and Coupland, 2004). Early grafting experiments revealed that the light signal is perceived in the leaves and is somehow transmitted to the apex where it induces flowering (Knott, 1934; Chailakhyan, 1936; Zeevaart, 1976). Until recently this signal, referred to as florigen, has been unknown. However, *FT* has been a likely candidate.

LD-induced flowering is dependent on the expression of the gene *CONSTANS* (*CO*) (Koornneef et al., 1991; Putterill et al., 1995). The expression of *CO* shows a circadian pattern (Suarez-Lopez et al., 2001). In SD conditions *CO* mRNA accumulation peaks during the night when it is dark. In LD conditions the peak in mRNA accumulation occurs at roughly the same time after dawn and the higher levels of *CO* mRNA hence coincides with light (Suarez-Lopez et al., 2001). The CO protein is rapidly degraded in dark (Valverde et al., 2004). In LD CO activates the expression of *FT*- and *SOC 1* mRNA (Yoo et al., 2005).

The main target of CO is *FT* (Wigge et al., 2005). *FT* is mainly expressed in the vascular tissue of leaves. In order for FT to induce flowering it integrates with FD; a transcription factor predominantly expressed in the shoot apex, even before flowering is induced (Abe et al., 2005; Wigge et al., 2005). The *FT* mRNA has now been found to move from leaves to the shoot apex where flowering is induced (Huang et al., 2005). The *FT* mRNA is therefore part of the intangible florigen. *FT* controls the timing of flowering and the protein-protein interaction between FT and FD at the shoot apex, mediates the flower initiation through the activation of *APETALA1* (*AP1*), a flower meristem identity gene (Wigge et al., 2005). *FT* and *SOC1* are both powerful activators of flowering; the

overexpression of these genes will cause extremely early flowering phenotypes in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000).

The *FT* ortholog in aspen trees has recently been shown to possess a similar function as that of Arabidopsis. Together with the *CO* ortholog it controls the timing of flowering in spring as day length increases, but also the cessation of growth in the autumn as day length decreases (Bohlenius et al., 2006). The flower initiation in forest trees is suggested to be activated when the *FT* expression exceeds a critical threshold value (Bohlenius et al., 2006). Forest tree breeding programs of today are highly limited by the generation time as compared to annual crops. The possibility to induce early flowering in forest trees and the knowledge of mechanisms controlling flower initiation as well as growth cessation might therefore be of great importance for the tree breeding programs of tomorrow.

Since the stability of CO protein is highly affected by light (Valverde et al., 2004), there is also reason to believe that light is of importance for *FT* expression. In order to study the function of *FT*, a heat inducible system has been used in this work. Transgenic *A. thaliana* with the construct *Hsp::FT Hsp::GUS* was generated. It has earlier been noticed that flower initiation induced by heat shock treatment, seems to be more efficient in light than in darkness (Henrik Bohlenius, unpublished). Therefore, the main objective with this work has been to investigate if there are any differences in the capability of flower initiation due to different light conditions, and if so, to investigate the causes of this variation. Another aim has been to investigate weather or not there is an optimal time for heat treatment in order to induce the *Hsp::FT Hsp::GUS* construct.

RESULTS

Flower induction by *Hsp::FT* requires light

It has earlier been noticed that flower induction with the heat inducible system *Hsp::FT* appears to be more efficient in light than in dark (H. Bohlenius, unpublished). In order to investigate this closer, *Hsp::FT* plants grown in SD were heat-treated in light and dark respectively and then returned to SD conditions. Four weeks after the treatment the amount of flowering plants was counted. Of the plants heat-treated in light, 84% were flowering as compared to 13% of the plants heat-treated in darkness (Fig. 1). None of the untreated controls were flowering. This result verified that the induction seems to be more efficient in light.

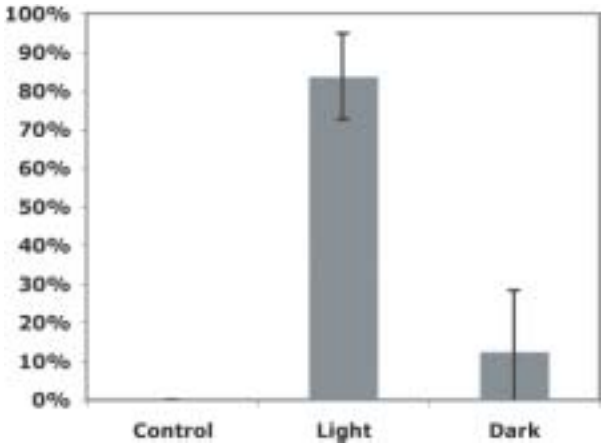

Figure 1. Efficiency of FT-induced flowering in light and dark conditions. Plant material carrying the heat inducible construct *Hsp::FT Hsp::GUS* were grown in SD conditions, heat treated for one hour at 42°C and 37°C respectively, and then returned to SD conditions. Data is shown as the percentage of flowering plants four weeks after treatment. Control: n=17, Light: n=17 and Dark: n=21. Error bars represent standard error.

The induction capability of the heat shock promoter is similar in light and darkness

In order to find out the reason for the difference in efficiency of flower induction, the activity of the heat shock promoter was investigated in light and in dark. Individual leaves of *Hsp::FT Hsp::GUS* plants were heat shock induced for different periods of time, ranging between 30 and 210 minutes, and analyzed for accumulation of *FT* and *GUS* mRNA.

Both *GUS* (Fig. 2A and B) and *FT* (Fig. 2C and D) mRNA accumulation reaches a peak after 60-90 minutes and then declines back to background level, indicating that this time interval is sufficient for heat shock induction of the *Hsp::FT Hsp::GUS* construct. These results are in agreement with earlier findings

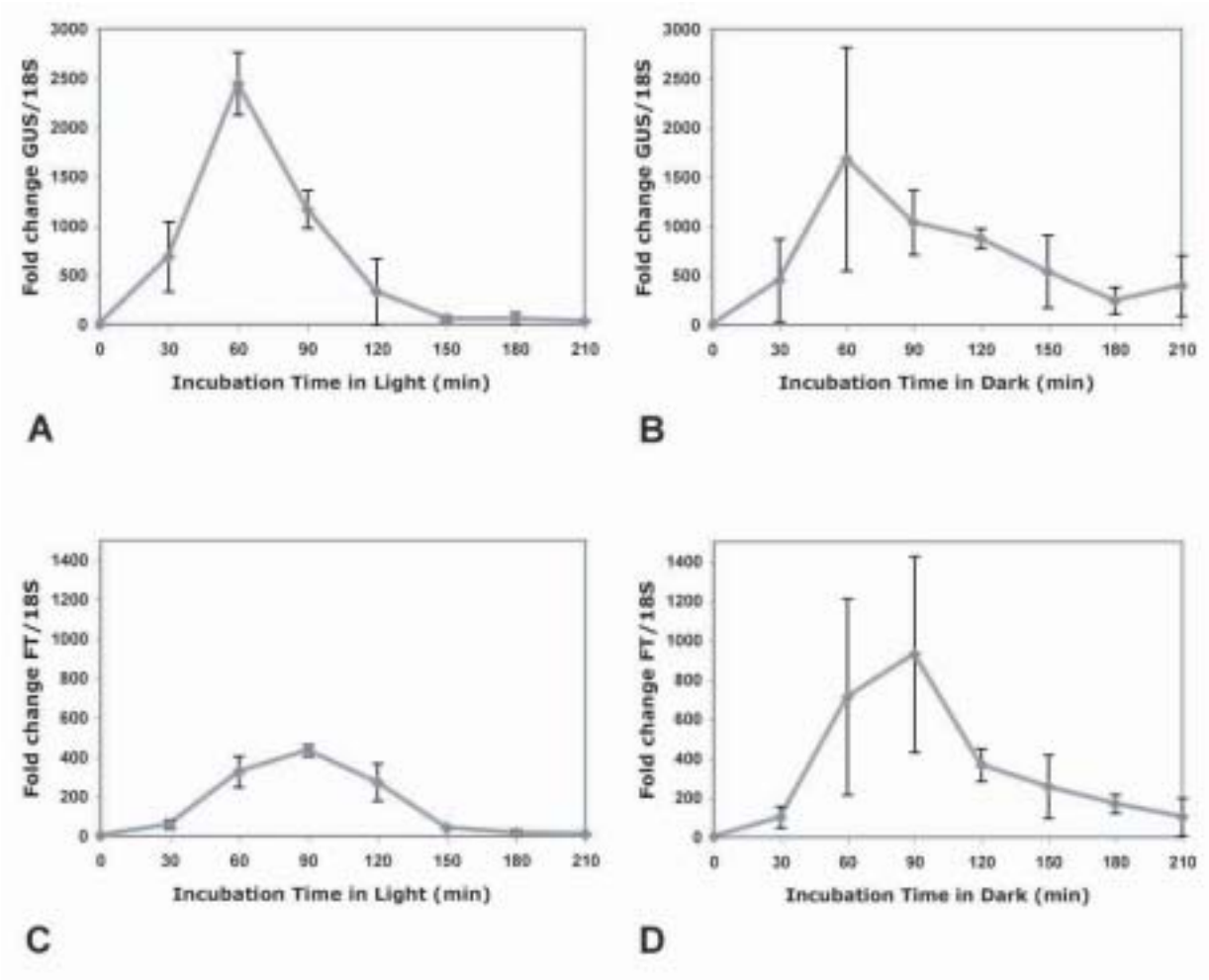

Figure 2. Heat shock induction of *GUS* and *FT* expression for different periods of time in light and darkness. *Hsp::FT Hsp::GUS* transgenic plants were grown in SD conditions for 21 days and then heat treated in light **(A, C)** or in darkness **(B, D)**. Samples were harvested every 30 minutes for 210 minutes and the *GUS* **(A, B)** and *FT* **(C, D)** mRNA was followed. The gene specific *FT* and *GUS* expression was normalized against *18S* and the minimum values were set to one. Error bars represent standard deviation. Three biological- and three technical replicates were used.

(Huang et al., 2005). The heat shock induction of *GUS* and *FT* shows that there is no difference in the induction kinetics or the relative fold induction between the two constructs in light and in dark (compare figs 2A and 2C with figs. 2B and 2D). This suggests that the expression of the heat-shock promoter is as efficient in dark conditions as in the light.

The translation efficiency of *GUS* mRNA is similar in light and in darkness

Another reason for why *Hsp::FT* is inefficient in inducing early flowering in the dark (Fig. 1) could be a poor general translational efficiency during dark conditions. In order to test this, heat-induced *Hsp::GUS* were analyzed with GUS activity stainings after induction in both light and darkness. The results show a comparable staining in both light and dark (Fig. 3), confirming that there is no difference in the transcriptional activity of the heat shock inducible promoter, nor the translation of the protein in light and darkness.

In order to further investigate, and also quantify the translational efficiency, the GUS activity in the *Hsp::GUS* plants was measured with a MUG assay. The results from two independent experiments show no indication of a higher activity in light (Fig. 4). On the contrary, *Hsp::GUS* induction in the dark lead to higher GUS activities than in the light (Fig. 4). These data rule out the possibility that translation in general works poorer in darkness than in light.

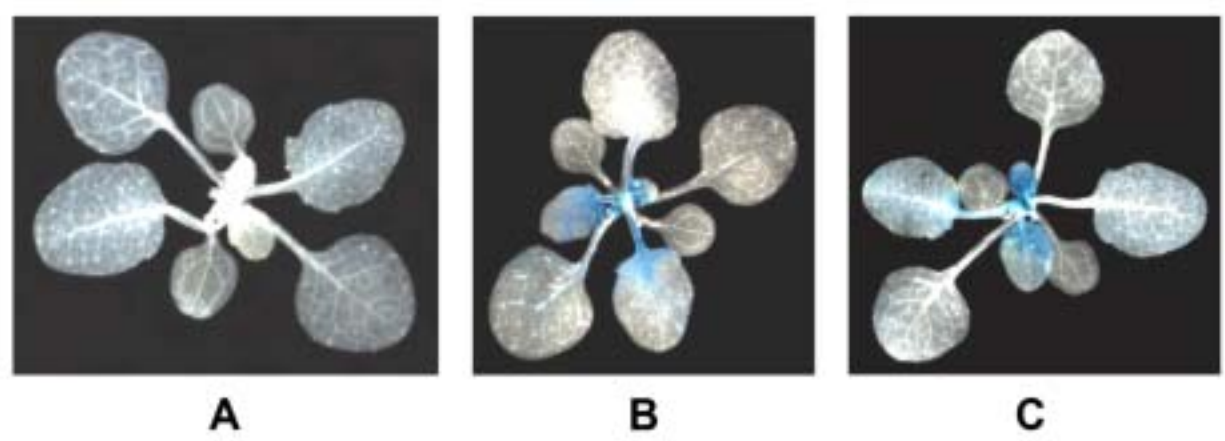

Figure 3. X-Gluc staining of GUS activity in *Hsp::FT Hsp::GUS* plants that have been heat shock-induced in light or darkness. **(A)** Non-heated control. **(B)** Heat shock performed in light conditions. **(C)** Heat shock performed in dark conditions.

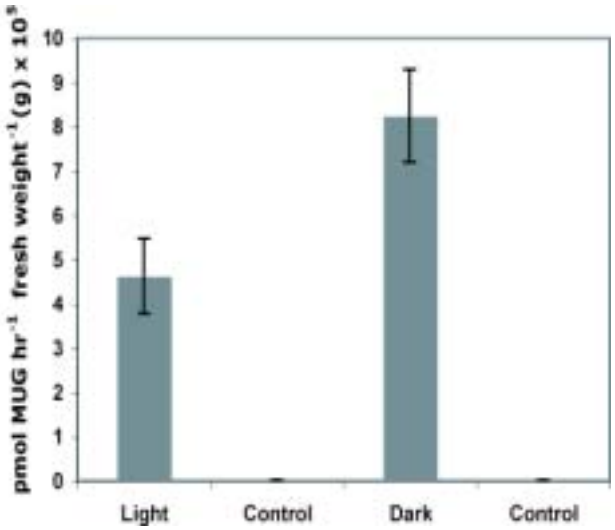

Figure 4. The GUS activity in heat shock treated single leaves of transgenic *Hsp::FT Hsp::GUS* Arabidopsis plants induced in either light or dark conditions. The experiment was carried out twice with similar results and technical duplicates were used for each of the experiments. 24 heat-shocked leaves and 6 non-treated control leaves were used for each of the experiments. Error bars represent standard error.

Light conditions do not affect the stability of the FT protein

Unpublished data suggest that *35S::FT* expressing hybrid aspen trees form more flowers under high-light than during low-light conditions (Bohlenius et al., in prep.) Since the CO protein has been shown to be very unstable in darkness (Valverde et al., 2004), one could also speculate that light could be of importance for the FT protein stability. Earlier studies demonstrate that in long days *FT* displays a diurnal expression pattern increasing at the end of day and peaking during the night (Suárez-López et al., 2001). This increase in *FT* expression is dependent on a high expression level of the upstream gene *CO* coinciding with light in the evening (Suárez-López et al., 2001; Valverde et al., 2004).

In order to study if the *35S*-driven *FT-HA* mRNA displays any diurnal variation in its accumulation and to investigate if the FT protein has a similar post-transcriptional regulation as the CO protein, transgenic *35S::FT-HA* plants that were grown in LD and SD were harvested with a time interval of four hours, starting at time point zero directly after night conditions.

A gene specific RT-PCR was carried out in order to analyze the *FT-HA* expression pattern. The results show that *FT-HA* accumulation in LD peaks at 20 h, and that the observations made by Suárez-López and colleagues for the endogenous *FT* mRNA accumulation, unexpectedly, also holds true for the transgenic *35S::FT-HA* transcript (Fig. 5A). In SD conditions however, the *FT-HA* expression remains constant during the 24 hours analyzed (Fig. 5 B). The FT protein accumulation in the same plant material was analyzed by Western blotting (Fig. 5 C-D). Surprisingly, the protein signal seems to demonstrate a similar pattern and intensity in both LD and SD, with a slightly stronger intensity the first eight hours of the light period. This suggests that the efficiency of *FT* translation or the stability of the FT protein can vary over the day, with a lower translational activity or lower stability during the night.

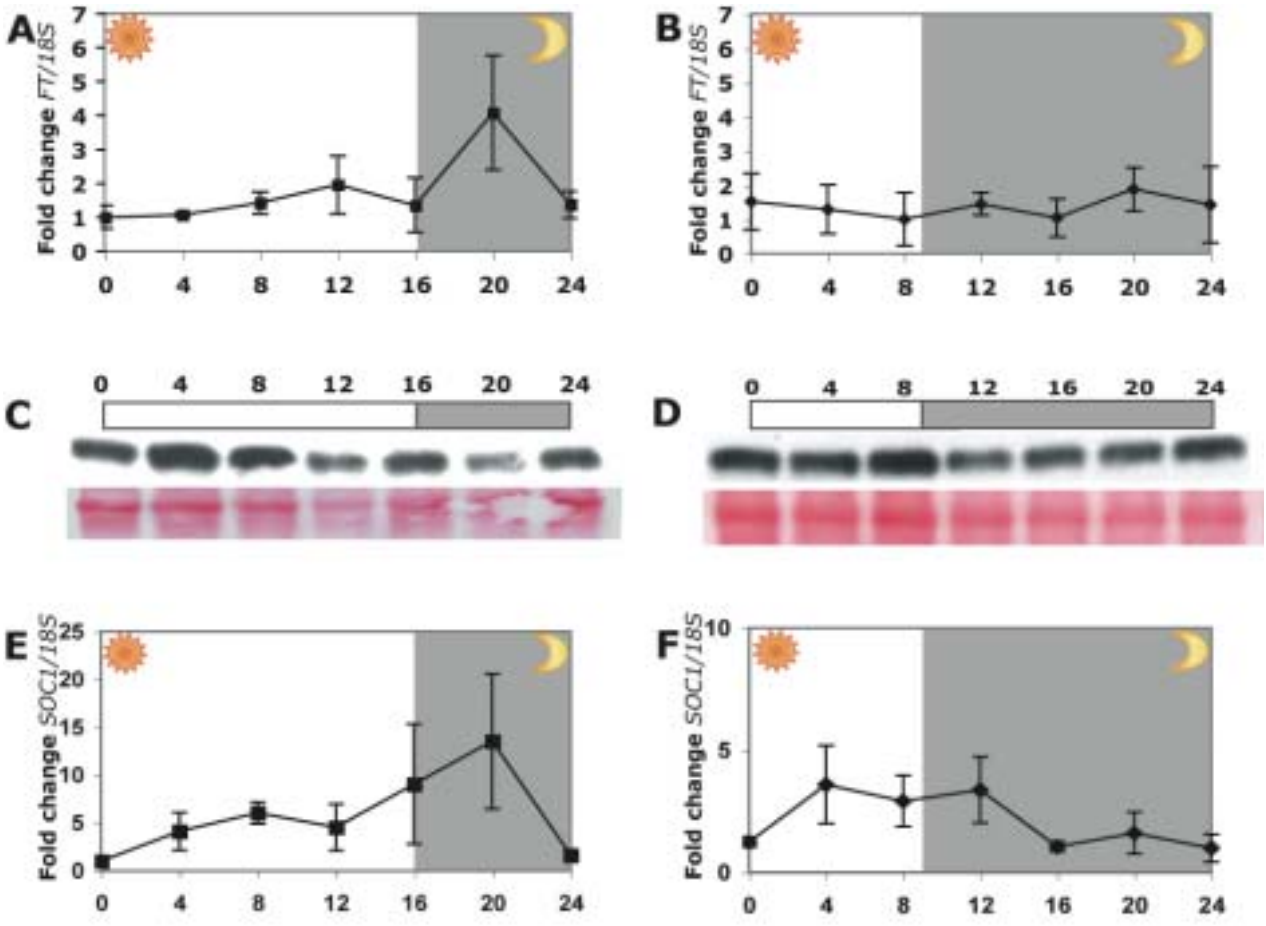

Figure 5. *FT-HA* and *SOC 1* expression and FT-HA protein accumulation in leaf extracts from four weeks old transgenic *35S::FT-HA* Arabidopsis plants grown in LD and SD. **(A)** *FT* expression in LD. **(B)** *FT* expression in SD. **(C)** FT-HA protein accumulation in LD analyzed by Western blotting. **(D)** FT-HA protein accumulation in SD analyzed by Western blotting. **(E)** *SOC1* expression in LD. **(F)** *SOC1* expression in SD. The gene specific *FT* and *SOC1* expression from LD and SD was normalized against *18S* and the minimum values were set to one. Error bars represent standard deviation. For the protein detection an equal amount of protein extract was loaded to each well of the gels. Time scale presented in ZT, Zeitgeber time starting directly after night conditions.

The diurnal expression of *SOC1*, a downstream target of *FT* and *CO*, was also analyzed, showing a similar expression pattern as that of *FT* with the exception of a slight increase between 4 h and 12 h in both LD and SD (Fig. 5 E-F).

Light quality does not affect FT protein stability

In *35S::CO* Arabidopsis plants, the stability of the CO protein has been shown to be sensitive to different light qualities (Valverde et al., 2004). The protein is unstable in darkness and in red light. In accordance with this, after exposure of plants to blue (B), far-red (FR) or white (W) light, higher amounts of *FT* mRNA were detected in nuclear extracts from *35S::CO* than for plants exposed to red (R) light or darkness (D) (Valverde et al., 2004).

In order to investigate if the FT protein responds to different light qualities, and if the protein expression fluctuates in a circadian manner, *35S::FT-HA* Arabidopsis plants grown in SD were placed in the different light qualities for one SD. Samples were harvested every four hours, commencing at time point zero directly after night conditions and Western blotting was performed in order to detect the protein. The results do not indicate any clear circadian fluctuations in the protein stability (Fig. 6 A-E). Valverde and colleagues treated the plant material in continuous light for two days prior to RNA extraction. In order to investigate if the time period of the light quality treatment influences how the plants react, plants were put

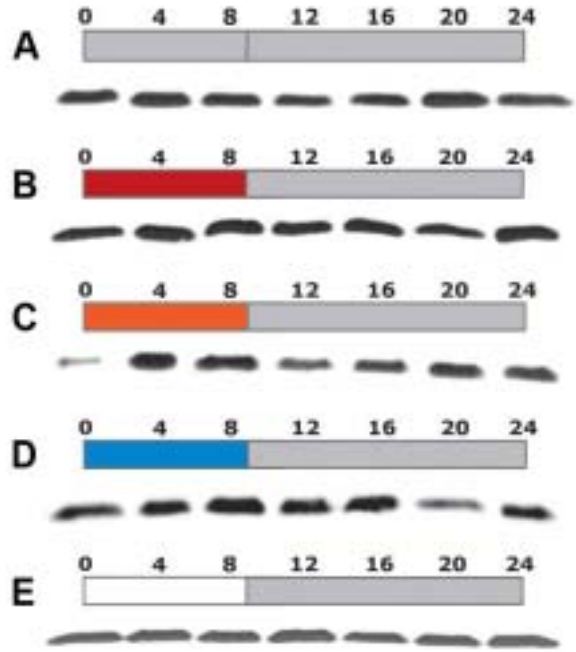

Figure 6. FT-HA protein accumulation in leaf extracts of three weeks old *35S::FT-HA* transgenic plants analyzed by Western blotting. **(A)** Darkness. **(B)** Far red light. **(C)** Red light. **(D)** Blue light. **(E)** White light. Time scale presented in ZT, Zeitgeber time, starting directly after night conditions. For the protein detection an equal amount of protein extract was loaded to each well of the gels.

Figure 7. FT-HA protein accumulation in leaf extracts of three weeks old *35S::FT-HA* transgenic plants analyzed by Western blotting after different time periods in D, Darkness; FR, Far red light; R, Red light; B, Blue light and W, White light. The plant material was treated with different light qualities for four hours **(A)** or 48 hours **(B)**. All samples were collected at noon. For the protein detection an equal amount of protein extract was loaded to each well of the gel.

in the different light qualities continuously for 48 hours. Samples were harvested at noon and analyzed by Western blotting together with the 4 h samples from the circadian light quality experiment. The same amount of protein extract was loaded to each of the wells on the same gel. No differences due to the time course of the treatment could be distinguished. The protein signal from the plant material treated in the different light qualities for four hours was as strong as that of the material treated for 48 hours (Fig. 7). Taken together, these data show that FT protein stability, in contrast to CO protein stability, is not regulated by differences in light conditions or light qualities.

DISCUSSION

The observation made by Bohlenius, that flower induction with the heat inducible system *Hsp::FT Hsp::GUS* is more efficient in light than in dark, was supported by the results from my experiment (Fig. 1). The reason for this apparent light dependent activity of *FT* was investigated in this work.

When individual leaves were heat induced in light and darkness, both *GUS* and *FT* reached a peak after 60-90 minutes and then declined back to background level. This indicates that there is no difference in the induction kinetics, or the relative induction levels, of the two constructs in light and in dark (Fig. 2) hence the expression of the heat-shock promoter is as efficient in both conditions. This was to be expected since there is no reason to believe that the heat shock promoter is affected by different light conditions, although one could not have ruled out the possibility that the transcriptional efficiency could have been lower in darkness.

In order to out rule the possibility that the protein translation efficiency in general is poorer in dark than in light, heat induced plants were assayed for qualitative and quantitative differences in the activity of the GUS reporter enzyme through x-Gluc stainings (Fig. 3) and GUS activity measurements with a MUG assay (Fig. 4). None of these experiments showed any indications of a higher GUS activity in light. This proved that translational efficiency in general is similar between dark and light conditions. However, this does not exclude the possibility that *FT* displays a specific post-transcriptional regulation by light, thus preventing flower induction in darkness.

Since the CO protein is not stable in darkness (Valverde et al., 2004), the peak in wild type *FT* mRNA expression between zeitgeber time 16-20 h has been contributed to the fact that in LD conditions, the CO protein is exposed to light (Suarez-Lopez et al., 2001; Valverde et al.,

2004). Furthermore, the increase of *FT* around 16 h coincides with a high expression level of *CO* (Suarez-Lopez et al., 2001; Valverde et al., 2004). This is consistent with the idea that in LD CO activates *FT*, which in turn promotes flowering through the activation of downstream targets such as *SOC 1* (Yoo et al., 2005).

The results from the *35S::FT-HA* experiments in LD in this work (Fig. 5A), suggests that the *FT* expression peak at approximately 20 h in wild type plants observed by (Suarez-Lopez et al., 2001), also holds true for the *35S::FT-HA* Arabidopsis plants. This is surprising since the 35S promoter was expected to drive the expression of *FT-HA* on a high and constant level in the same manner as it drives the *CO* RNA expression in transgenic *35S::CO* (Valverde et al., 2004). This suggests a diurnal variation in post-transcriptional regulation of *FT* mRNA stability, and suggests that the CO-dependent peak in *FT* expression at the end of the day could be caused by a combination of transcriptional activation and a specific induction of *FT* mRNA stability. The mechanism influencing the peak of the *35S::FT-HA* expression is yet to be discovered, however the peak observed in wild type plants fails to appear in *co* mutant background (Mouradov et al., 2002). Therefore it would have been interesting to repeat the same experiment in *35S::FT-HA co* double mutants, and also to study if there is a difference in the peak of GUS mRNA accumulation in a *FT::GUS* reporter gene construct.

The initiation of flowering in *Arabidopsis thaliana* is mediated by different pathways, amongst these are the photoperiod response pathway, which includes the onset of *CO* in response to LD, which in turn activates *FT* and *SOC 1*. Additionally, *FT* and *SOC 1* are regulated by other environmental cues such as exposure to low temperatures, but also exposure to plant hormones such as gibberellins, as the pathways converges into an integrating pathway (Mouradov et al., 2002). Some of these environmental cues or some other yet unknown functions might also be involved in regulating the stability of the *FT* transcript in LD. In SD conditions, the gene specific *FT-HA* expression remains constant as expected (Fig. 5 B). This result makes it tempting to assume that the mechanism influencing the peak of *35S::FT-HA* transcript in LD is associated with CO stability and the photoperiod response pathway.

Since the results from the analysis of *FT* transcription and mRNA stability indicates that, if anything, transcriptional activity and *FT* mRNA accumulation is higher in darkness than in the light, it is obvious that this can not be the explanation for the reduced efficiency of FT-induced floral induction in the dark. Therefore, the observations were extended to also look at the stability of the FT protein under different light conditions and light qualities. Surprisingly, the FT-HA protein signal seems to demonstrate a similar pattern and intensity in both LD and SD irrespective of the expression pattern of the transcript (Fig. 5 C-D), with a slightly stronger intensity the first eight hours of the light period. Unfortunately the Ponceau staining performed in order to assure that the same protein amount was loaded displayed a similar tendency. However, this might be caused by a putatively circadian fluctuation of Rubisco, the most abundant protein in plants. Circadian oscillations with a peak soon after dawn in the RNA expression of both *RCA* (the gene encoding Rubisco activase) and *RBCS* (the gene encoding the small subunit of Rubisco) have been shown in Arabidopsis grown in 14 h of light and 10 h of darkness (Pilgrim and McClung, 1993). Hence one may question the accuracy of using total protein amount as a loading control in a circadian experiment. However, it is also clear that the peak in *FT-HA* mRNA accumulation during darkness in LD, is not matched by any increase in FT-HA protein accumulation (Fig. 5A and C), this might therefore indicate a reduced FT-HA translational efficiency or protein stability during darkness or an increased efficiency or protein stability during light. If this would also extend to the FT protein, this could be a mechanism that, at least partly, can explain the reduced efficiency of FT-induced flowering in darkness.

The expression of *SOC1*, a downstream gene of *CO* and *FT*, demonstrates a similar expression pattern as that of FT-HA with a slight increase

between 4 h and 12 h in both LD and SD (Fig. 5 E-F). The slight increase might be due to the stronger FT-HA protein signal at roughly 4-8 h in both LD and SD (Fig. 5 C-D), while the later, more pronounced peak in LD (Fig. 5E) is probably caused by the same mechanism that controls the peak in *FT-HA* expression.

Valverde and colleagues clearly showed that the CO protein in *35S::CO* Arabidopsis plants is unstable in darkness and red light. I investigated if the FT-HA protein also responds to different light qualities, and if the protein expression fluctuates in a circadian manner. No clear circadian fluctuations of the protein stability (Figure 6 A-E), nor any differences due to the time course of the light quality treatment could be distinguished (Fig. 7). This indicates that the FT-HA protein is not regulated in the same manner as CO, since CO is rapidly degraded in darkness and red light.

The results from the white light experiment (Fig. 6 E), does not seem to support that of the circadian experiment earlier performed in SD where a lower protein accumulation was detected in the night (Fig. 5 D). However, this might simply be due to a more equal loading of protein in the white light experiment, or it might be due to the younger material used in this experiment.

It should be emphasized that these experiments were conducted on plants grown in SD. It would have been interesting to investigate plant material grown in LD as well. It should also be taken into consideration that Valverde and colleagues used protein from nuclear extract, while in this experiment extract from whole leaves was used. FT interacts with FD in the nucleus of shoot apex, but FT has also been localized to the cytoplasm (Abe et al., 2005). If it is so that the FT protein moves between different cell compartments, my results do not reflect the presence of protein in the nucleus at a given time, but the presence of protein in the leaf tissue as a whole. Sub-cellular localization of an FT-GFP fusion protein could have been performed in order to trace the protein at the same time intervals.

To conclude, I have shown here that *FT* expression might be subjected to a rather complex posttranscriptional regulation possibly involving effects on both *FT* mRNA stability and the efficiency of translation or stability of the FT protein. This post-transcriptional regulation might contribute to the reduced efficiency of FT-induced flowering in darkness as previously suggested by Bohlenius, and also verified here. However, it is also possible that this difference could be attributed to a completely FT-independent process. One could for instance envision changes in the general efficiency of the phloem-mediated mRNA loading or transport between light and darkness, or in the ability of the *FT* mRNA to reach target tissues in the shoot apex. This possibility could be tested by following the movement of the *FT* mRNA from leaf to shoot apex, comparing light and dark conditions.

METHODS

Plant Material

For GUS staining, flower induction experiments and GUS activity experiments *Arabidopsis thaliana Hsp::FT Hsp::GUS* plants were used (Huang et al., 2005). For circadian experiments, i.e. RNA extraction, cDNA synthesis, RT-PCR and Western blotting, *35S::FT-HA* plant material was used. For circadian experiments in different light qualities *35S:FT-HA* plant material was used. All experiments were performed in the ecotype Columbia.

Growth Conditions

Seeds were vernalized in darkness for a minimum of one day at 8°C in 0.1% agarose before sowing. Plants were grown at 23°C in long- or short day conditions (LD- 16 h of light and 8 h of darkness and SD- 9h of light and 15 h of darkness). The light intensity in both LD and SD conditions was approximately 140μmol $m^{-2} s^{-1}$ and Philips Master TLD58W/840 fluorescent lights were used as light source.

RNA Extraction and RT-PCR

Plant tissue was put in 2ml eppendorf tubes and frozen in liquid nitrogen. A metal bead was added to each tube before the tissue was grinded into a fine powder in a bead mill (maximum speed, 15 sec).
The RNA extractions were performed as described in the Aurum™ Total RNA mini kit from BIO RAD. The quality of the RNA extractions was reassured by running the samples on a 1.5% agarose gel.
cDNA synthesis was performed as described in the iScript™ cDNA Synthesis Kit (BIO RAD). The amount of total RNA for the cDNA synthesis varied between

85-125ng. RT-PCR reactions were performed as described in the iQ™ SYBR® Green Supermix, (BIO RAD). Quantification was performed on iCycler iQ RT-PCR from the same manufacturer. The results were normalized to 18S rRNA expression and for the circadian experiments the lowest value was set to one. Three biological- and three technical replicates were used for every sample point. For RT-PCR reactions, the following primers were used:
18S 5'- TCAACTTTCGATGGTAGGATAGTG,
18S 3'- CCGTGTCAGGATTGGGTAATTT,
GUS 5'- AACGGCAAGAAAAAGCAGTC,
GUS 3'- TGATATCGTCCACCCAGGTG,
FT 5'- GGCATCGTATCAAGCTTACTAGTG,
FT 3'- CCTTAGATCCAAGCCATTAGTCAC,
SOC1 5'- CAACAGATTGAGCAACAGCTTGAG,
SOC1 3'- AGCTTCTCGTTTTCTGCAGCTAG.
The annealing temperature used for *18S*, *FT* and *GUS* was 52°C and for *SOC1* 55°C. The number of cycles for all genes was set to 40.

Heatshock Experiments

For flower induction, whole plants grown in SD conditions were heat treated for one hour in light and darkness at 42°C and 37°C respectively. After the treatment the plants were moved back to SD conditions and flowering plants were counted after four weeks.
For heat shock induction of GUS and FT expression, whole plants grown in SD conditions were heat treated for different periods of time ranging between 30 and 210 minutes. Plant material was harvested every 30 minutes.

X-Gluc Staining of GUS Activity

Whole plant material stored in pots, water and x-Gluc solution was induced for 0, 1, 2, and 3h. The dark- and light samples were induced in 37°C and 42°C respectively. Plant material was put in normal temperature conditions (23°C) in darkness and light respectively for 2 hours before plant tissue was collected and GUS staining was performed according to protocol (Weigel and Glazebrook, 2002) with a few modifications. Infiltration of the samples was performed with and without vacuum. The samples were incubated at 37°C over night and then treated with FFA (5% formaldehyde, 5% acetic acid, 20% EtOH) for 10 minutes, 50% EtOH for 2 minutes and 100% EtOH for >10 minutes. Samples were stored in 100% EtOH.

GUS Activity

Individual leaves were placed on a copper plate connected to a hot water bath and heated to 37°C in light and darkness respectively for one hour. Each leaf was grinded and mixed with 300µl GUS buffer (50mM sodium phosphate buffer pH 7, 10mM EDTA, 0,1% SDS, 0,1% triton-X 100). The samples were centrifuged at 14000rpm for 3 minutes and 25µl of the supernatant was removed and added to 175µl 1mM 4-MUG in GUS buffer. The samples were incubated for 2 hours at 37°C and the reaction was blocked by the addition of 100µl 1M Na_2CO_3 to each sample. Duplicates of 130µl from the samples were transferred to a 96 well microtiter plate. Quantification of the GUS activity after heat treatment was performed by measuring the fluorescence from 4-methyl umbelliferone (4-MU) with a spectrofluorometer (Spectra MAX Gemini) at 455nm. As a substrate 4-methyl umbelliferryl glucuronide (4-MUG) was used. The GUS enzyme will convert the 4-MUG into the fluorescing product 4-MU; hence the fluorescence can be measured. The experiment was carried out twice with similar results, 24 heat-shocked leaves and 6 non-treated control leaves were used for each of the experiments.

Protein Extractions SDS-PAGE and Western Blotting

Plant material was grinded in liquid nitrogen. 25mg plant tissue was mixed with150µl Tris-Glycine SDS Sample buffer in 1.5 ml eppendorf tubes and heated to 95°C for 10 minutes in order to denature the proteins. The supernatant was removed from the samples. A 16% Tris-Glycine Gel from Invitrogen was used for the circadian experiments and 15% Tris-HCl gels from BIO RAD were used for the circadian light quality experiments as well as the continuous light quality experiment. An equal volume of protein extract was loaded to the wells. Running buffer (25mM Tris, 200mM Glycine, 0.1% SDS) was added to the chamber of the XCell *SureLock*™ Mini-Cell (Invitrogen) and Mini Protean® 3 Cell (BIO RAD). The gels were run at 200V for approximately 1h. The transfer was performed at 20V constant voltage for 1h and then at 25V for 45 minutes, using transfer buffer (50mM Trisbase, 50mM Boric acid). The membranes (Immobilon-P PVDF transfer membrane Millipore IPVH00010) were stained in 0.2% Ponceau S (Serva) in 3% TCA for 3 minutes and destained in 1% acetic acid. After scanning, the membranes were washed in TBST (50mM Tris-HCl, 150mM NaCl, 0.05% Tween-20) and blocked over night in blocking solution (TBST+5% milk powder). Membranes were washed in 50ml TBST and incubated with primary antibody for 2 hours on a shaker. (Rabbit polyclonal to HA tag- ChlP Grade ab9110-100 from Abcam, diluted 1:4000 with blocking solution). The membranes were washed with TBST, blocking solution diluted two times with TBST, TBST and finally with MQ water, for 10 minutes each. They were then incubated with the secondary antibody for 1 hour (Peroxidase labeled anti-rabbit lgG (H+L) from VECTOR, diluted 1:20 000 with blocking solution). The same washing procedure as for the primary antibody was performed. Membranes were incubated in ECL reagent as recommended by manufacturer (Amersham Biosciences) and exposed to a film (AGFA Cronex 5 Medical x-ray film). The experiments were repeated with similar result.

Circadian Light Quality Experiment

Three weeks old plants grown in SD conditions were placed in different light qualities; dark, far-red, red, blue

and white light for one SD. Leaves were harvested and put in liquid nitrogen every 4 hours for 24 hours, starting at time point 0 ZT, directly after night conditions. The light intensity was 20μmol $m^{-2}s^{-1}$ for all light qualities.

Continuous Light Quality Experiment

Three weeks old plants grown in SD conditions were placed in continuous light of different qualities as described above. Leaves were harvested at noon after 48 hours of continuous light treatment and used for protein extraction and Western blotting.

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