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Strawberry homologue of TERMINAL FLOWER1 integrates photoperiod and temperature signals to inhibit flowering

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SUMMARY

Photoperiod and temperature are major environmental signals affecting flowering in plants. Although molecular pathways mediating these signals have been well characterized in the annual model plant *Arabidopsis*, much less information is known in perennials. Many perennials including the woodland strawberry (*Fragaria vesca* L.) are induced to flower in response to decreasing photoperiod and temperature in autumn and they flower following spring. We showed earlier that, in contrast with *Arabidopsis*, the photoperiodic induction of flowering in strawberry occurs in short days (SD) when the decrease in *FvFT1* (*FLOWERING LOCUS T*) and *FvSOC1* (*SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1*) expression leads to lower mRNA levels of the floral repressor, FvTFL1 (TERMINAL FLOWER1). By using transgenic lines and gene expression analyses, we show evidence that the temperature-mediated changes in the *FvTFL1* mRNA expression set critical temperature limits for the photoperiodic flowering in strawberry. At temperatures below 13°C, low expression level of *FvTFL1* in both SD and long days (LD) allows flower induction to occur independently of the photoperiod. Rising temperature gradually increases *FvTFL1* mRNA levels under LD, and at temperatures above 13°C, SD is required for the flower induction that depends on the deactivation of *FvSOC1* and *FvTFL1*. However, an unknown transcriptional activator, which functions independently of FvSOC1, enhances the expression of *FvTFL1* at 23°C preventing photoperiodic flowering. We suggest that the observed effect of the photoperiod × temperature interaction on *FvTFL1* mRNA expression may allow strawberry to induce flowers in correct time in different climates.

Keywords: flowering, temperature, photoperiod, TERMINAL FLOWER1, *Fragaria vesca*, strawberry, Rosaceae.

INTRODUCTION

Photoperiod and temperature are essential signals for plants to adjust their growth and development. Photoperiod serves as a stable indicator of the season, whereas temperature as a variable environmental parameter is less reliable seasonal cue. However, the relative importance of these signals depends on the species. Photoperiod is a major signal affecting seasonal development in perennial trees, and strong latitudinal clines in their photoperiodic responses have been observed (Heide, 1974; Böhlenius *et al.*, 2006). However, other perennials monitor both photoperiod and temperature to induce flowering, growth cessation, bud set or dormancy (Junttila, 1980; Thomas and Vince-Prue, 1997; Junttila *et al.*, 2003; Heide and Sønsteby, 2007; Heide, 2008). In apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.), in contrast, decreasing temperature instead of photoperiod determines the timing of growth cessation and dormancy induction (Heide and Prestrud, 2005).

FLOWERING LOCUS T (FT), which belongs to the family of phosphatidyl ethanolamine binding proteins (PEBP), is a major photoperiodic signaling molecule, a mobile protein called florigen, in plants (Corbesier *et al.*, 2007; reviewed by Pin and Nilsson, 2012). In the photoperiodic flowering pathway of *Arabidopsis thaliana* (L.) Heynh., long days (LD) stabilize CONSTANS (CO) protein that is encoded by rhythmically expressed *CO* mRNA (Valverde *et al.*, 2004). In leaves, CO activates the expression of *FT* (Samach *et al.*, 2000; Takada and Goto, 2003). Then, FT protein moves through the phloem to the shoot apical meristem and forms a complex with bZIP transcription factor FD to activate the floral meristem identity genes (Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Jaeger and

Wigge, 2007; Tamaki *et al.*, 2007). FD also interacts with another PEBP, TERMINAL FLOWER1 (TFL1), which represses flowering although it shows high similarity to FT (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006; Hanano and Goto, 2011). After flower induction has occurred, *TFL1* is highly expressed in the inflorescence meristem to maintain its indeterminate state (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1997; Ratcliffe *et al.*, 1999).

In perennial plants, molecular studies on the timing of flowering and bud set have been focused on *FT* and *TFL1* like genes. In *Populus* trees, FT determines the timing of growth cessation and bud set, that occurs under SD in autumn (Böhlenius *et al.*, 2006). Hsu *et al.* (2011) showed evidence that two FT paralogs, FT1 and FT2, coordinately time developmental growth rhythm in *Populus.* It was suggested that FT1 promotes reproductive and FT2 vegetative development, and that FT2 is likely responsible for the timing of the growth cessation and bud set (Hsu *et al.*, 2011; Pin and Nilsson, 2012). Similarly, *FT/TFL1*-like gene induces bud set also in Norway spruce *Picea abies* (L.) H. Karst. (Karlgren *et al.*, 2013). We recently showed that in the perennial short-day (SD) plant woodland strawberry (*Fragaria vesca* L.), FvFT1 activates the homologue of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (FvSOC1)* specifically under LD (Mouhu *et al.*, 2013; Rantanen *et al.*, 2014). The expression of *FvFT1* and *FvSOC1* correlates negatively with flowering because the MADS domain transcription factor FvSOC1 activates the mRNA expression of the strong floral repressor FvTFL1 in the shoot apex (Koskela *et al.*, 2012; Mouhu *et al.*, 2013). TFL1 homologues function as major repressors of flowering also in other perennials including apple (Kotoda *et al.*, 2006; Flachowsky *et al.*, 2012), pear (Freiman *et al.*, 2012), rose (Iwata *et al.*, 2012) and *Populus* (Mohamed *et al.*, 2010).

In many species including some perennials, several weeks of cold temperatures increase the sensitivity to the flower-inductive signals in the apical meristem, the phenomenon known as vernalization. A MADS domain transcription factor FLOWERING LOCUS C (FLC) is responsible for vernalization requirement in *Arabidopsis* (Michaels and Amasino, 1999). FLC delays flowering by repressing the expression of floral promoters including *FT* and *SOC1*, and vernalization is needed to silence its expression (Helliwell *et al.*, 2006; Li *et al.*, 2008). Similarly, in the perennial *Arabis alpina* L. and *Cardamine flexuosa* With., the vernalization requirement is caused by FLC orthologs, PEP1 and CfFLC, respectively (Wang *et al.*, 2009; Zhou *et al.*, 2013). In *A. alpina*, two *FLC* like genes, *PEP1* and *PEP2*, are present, and PEP2 enhances the expression of *PEP1* (Bergonzi *et al.*, 2013). Also AaTFL1 is involved in the vernalization response in *A. alpina* by extending the vernalization period needed before flower induction can occur (Wang *et al.*, 2011). In *Arabidopsis*, the trimethylation of lysine 27 of histone 3 (H3K27me3) causes stabile silencing of *FLC* (Zhou *et al.*, 2013), whereas in *A. alpina*, vernalization transiently increases the level of H3K27me3 in the *PEP1* chromatin and decreases its expression allowing floral development to occur in the spring (Wang *et al.*, 2009; Zhou *et al.*, 2013).

Apart from vernalization responses, molecular understanding on the effect of the temperature on flowering is lacking in perennials. In *Arabidopsis*, FT mediates ambient temperature responses, and already a modest temperature change of 2–3°C increases its expression and accelerates flower induction (Blazquez *et al.*, 2003; Balasubramanian *et al.*, 2006; Strasser *et al.*, 2009; Kumar *et al.*, 2012). Ambient temperature pathway became established when *FCA* and *FVE*, the genes of the autonomous pathway, were observed to affect flowering time in response to ambient temperature (Blazquez *et al.*, 2003). Later, SHORT VEGETATIVE PHASE (SVP), a MADS domain floral repressor, was found to function downstream of FCA and FVE in the ambient temperature pathway (Lee *et al.*, 2007). In the *svp* mutant the expression level of *FT* is elevated, and based on chromatin immunoprecipitation studies, Lee *et al.* (2007) concluded that SVP binds to the *FT* promoter.

Detailed mechanism, how SVP controls *FT* expression in response to temperature, is emerging (Verhage *et al.*, 2014). Posé *et al.* (2013) showed evidence that the complex formation between alternatively spliced variants of FLOWERING LOCUS M (FLM) and SVP mediates the effect of the temperature on flowering time. At lower temperatures, splicing variant FLMβ dominates and forms a repressor complex with SVP (Lee *et al.*, 2013; Posé *et al.*, 2013). In consequence, the FLMβ-SVP complex binds to the promoter region of *FT* to repress its transcription. When temperature rises, FLMδ dominates compared with FLMβ. As the FLMδ-SVP complex does not bind *FT* chromatin, increased temperature allows the activation of *FT* transcription (Posé *et al.*, 2013). Also the destabilization of SVP at higher temperatures contributes to the activation of *FT* (Lee *et al.*, 2013), and Lee *et al.* (2014) suggested that the degradation of SVP protein instead of alternative splicing of *FLM* is a major mechanism in the ambient temperature signaling.

The role of histone variant H2A.Z in the temperature regulation of transcription is conserved in plants and yeast. The occupancy of H2A.Z in the nucleosomes is dynamically decreased by increasing temperature allowing RNA polymerase to bind gene promoters (Kumar and Wigge, 2010). H2A.Z is enriched also in the *FT* promoter at cool temperatures. At higher temperatures, decreased occupancy of H2A.Z nucleosomes on the *FT* promoter allows the binding of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) resulting in the activation of *FT* expression and accelerated flowering (Kumar *et al.*, 2012).

Many species of the Rosaceae family show temperature-dependent photoperiodic responses. The interaction of photoperiod and temperature has been shown to induce

flowering in raspberry (*Rubus idaeus* L.) and strawberry (Williams, 1960; Heide, 1977; Heide and Sønsteby, 2007) and dormancy in *Prunus* sp. (Heide, 2008). In the woodland strawberry (called as strawberry hereafter), decreasing temperature and shortening photoperiod induces flowering in autumn, flower initiation continues until the winter, and inflorescences outgrow in the following spring. In this species, temperatures above 20°C prevent flower induction, and SD is needed for the induction at intermediate temperatures, whereas cool temperatures can replace the photoperiodic requirement (Heide and Sønsteby, 2007). Similar photoperiod × temperature interaction has also been observed in several cultivars of the cultivated strawberry, *F.* × *ananassa* Duch. (e.g. Ito and Saito, 1962; Heide, 1977), but some cultivars do not show the temperature response (Sønsteby and Heide, 2006). Here we have studied at the molecular level how the interaction of photoperiod and temperature induce flowering in strawberry. Our results suggest that the temperature-mediated changes in the *FvTFL1* mRNA level set the critical temperature limits for the flower induction. The FvSOC1-dependent photoperiodic flowering only occurs between these temperature limits, and below the lower limit, the suppression of *FvTFL1* leads to the photoperiod-independent flower induction.

RESULTS

SD requirement of flower induction disappears below 13°C and above 20°C

Strawberry is a facultative short-day (SD) plant, in which temperature has a great influence on flower induction. To define critical temperature limits for the photoperiodic flower induction, we subjected strawberry seedlings to temperature series of 10, 13, 16, and 23°C under 12 and 18 h photoperiods (SD and LD, respectively). Both photoperiod and temperature affected flower induction. Cool temperature (10°C) induced flowering under both SD and LD conditions (Table 1). At 13°C, flowering occurred in both photoperiods, but one-third of the plants remained vegetative under LD indicating that the critical temperature that is low enough to fully replace the SD requirement is between 10 and 13°C. Furthermore, at 16°C, similarly to 18 and 20°C in previous studies (Heide and Sønsteby, 2007; Mouhu *et al.*, 2013), SD was obligatory for flower induction, whereas no flowering was observed at 23°C. In flowering plants, temperature had a significant effect on the days to flower and on the number of leaves in the primary shoot before terminal inflorescence ($P < 0.001$), whereas photoperiod had no effect on these parameters (Table 1). Plants had less leaves in the primary leaf rosette at 10°C than at higher temperatures (Tukey's test; $\alpha = 0.01$), but plants flowered earlier at 13 and 16°C compared with the lowest temperature (Dunn's test; $\alpha = 0.01$).

High temperature increases *FvTFL1* mRNA level

Our earlier studies indicated that the induction of *FvFT1* expression in strawberry leaves under LD activates *FvTFL1* through FvSOC1 in the shoot apex to repress flowering (Koskela *et al.*, 2012; Mouhu *et al.*, 2013). Therefore, we studied the expression of these genes after 4 weeks of different temperature-photoperiod combinations. In the leaves, *FvFT1* was strongly expressed only under LD and no expression was found under 12 h SD (Figures 1a,b and S1). However, temperature had only minor effect on its expression. We found highest expression levels at 16 and 19°C, but the *FvFT1* mRNA level tended to be lower at 23°C (Figures 1a,b and S1). In the shoot apex, the expression of *FvSOC1* was affected by both photoperiod and temperature. *FvSOC1* mRNA level decreased under SD compared with LD at all temperatures, but this photoperiodic effect was stronger at higher temperatures. In SD, *FvSOC1* mRNA level was reduced by half at the highest temperature compared with other temperatures, and in LD, its mRNA level tended to decrease at the highest and lowest temperatures (Figures 2a and S4a).

In contrast to the expression of *FvFT1* and *FvSOC1*, *FvTFL1* mRNA level correlated with flowering response in all temperature-photoperiod combinations. The expression level of *FvTFL1* was low at 10°C in both photoperiods, and under LD, gradual increase in its mRNA level with raising temperature was detected (Figure 2b). SD strongly suppressed *FvTFL1* at 16°C, as previously reported at 18°C (Mouhu *et al.*, 2013), and two-fold reduction on FvTFL1 mRNA level was also observed under SD at 13°C. Notably, under SD, *FvTFL1* mRNA levels gradually decreased with increasing temperature up to 16°C, whereas several-fold activation was found at the highest temperature.

We also analysed the expression of the floral marker genes *FvFUL1* and *FvAP1* (Koskela *et al.*, 2012) and mRNA levels of the strawberry *FD* homologue encoding a putative binding partner of both FT- and TFL1-like proteins (Abe *et al.*, 2005; Wigge *et al.*, 2005; Hanano and Goto, 2011) in the shoot apex. The expression level of *FvFD* was not affected by the photoperiod, but a two-fold lower mRNA level was observed at 10°C compared with higher temperatures (Figure 2c). *FvFUL1* was clearly activated in flower-inductive SD treatments at temperatures 10–16°C compared with non-inductive conditions (Figure 2d and Table 1). However, at the time point analysed in this experiment (4 weeks), no clear activation of *FvFUL1* was found under LD at 10 or 13°C, although also these treatments induced flowering. Moreover, another floral marker, *FvAP1*, was strongly induced only under SD at 13°C (Figure S2). Taken together, these data indicate that temperature-mediated changes in *FvTFL1* mRNA level set critical temperature limits for flower induction. Suppression of *FvTFL1* expression at cool temperatures probably

Table 1 Strawberry flowering responses to different temperature-photoperiod combinations. Percentage of flowering plants, the number of leaves in the primary leaf rosette before terminal inflorescence, and days until anthesis are shown. Plants were grown under indicated conditions for 6 weeks followed by flowering observations in a greenhouse. Values are means of 11–17 plants for 10 and 23°C treatments or 14–20 plants for 13 and 16°C treatments ± standard deviation

Temperature (°C)	Photoperiod (h)	Flowering plants (%)	Leaves before inflorescence[a]	Days until anthesis[a]
10	12	100	11 ± 1.2	46 ± 1.8
	18	100	10 ± 0.8	48 ± 1.6
13	12	91	13 ± 1.6	39 ± 12
	18	67	14 ± 1.3	45 ± 0
16	12	80	14 ± 1.3	36 ± 5.8
	18	0	–	–
23	12	0	–	–
	18	0	–	–

[a]Leaves before inflorescence and days until anthesis were counted only for flowering plants.

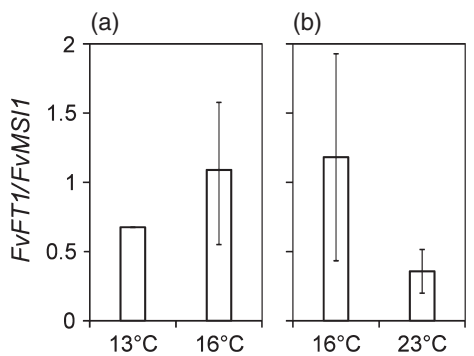

Figure 1. The expression of *FvFT1* in the leaves of strawberry plants grown at different temperatures.Two separate experiments at temperatures 13 and 16°C (a), and 16 and 23°C (b) were carried out under both short-day (SD) and long day (LD) conditions. Data for SD treatment are not shown, since the expression of *FvFT1* was detected only under LD. Samples were collected 16 h after dawn on 28th day after the beginning of the treatments. Values are means of three biological replicates ± standard deviation.

allows flower induction to occur. At intermediate temperatures, however, SD is needed for the *FvFT1*/*FvSOC1*-dependent reduction of *FvTFL1* mRNA level, whereas above the critical temperature limit, *FvTFL1* is highly expressed even under SD suppressing photoperiodic flowering.

Critical photoperiod for flowering is between 14 and 16 h

Heide and Sønsteby (2007) observed that the critical day length for flower induction in several Norwegian strawberry accessions is around 15 h. Therefore, we studied the effect of 14 and 16 h photoperiods on flower induction and the expression of flowering genes at 18°C. All plants flowered after 14 h photoperiod treatment, but 16 h photoperiod inhibited flower induction (Figure 3a,b). The expression of flowering time genes again correlated with the flowering response. *FvFT1* was strongly suppressed in 14 h photoperiod compared with the longer photoperiods (Figures 3c and S1). In parallel, we detected a clear reduction in the expression of *FvSOC1* and *FvTFL1* in the shoot apex in 14 h photoperiod (Figure 3c). Given that FvFT1 enhances *FvSOC1* expression in LD, which in turn, leads to higher mRNA expression of the floral repressor FvTFL1 (Mouhu *et al.*, 2013; Rantanen *et al.*, 2014), our results are in line with the hypothesis that FvFT1-FvSOC1-FvTFL1 pathway inhibits flower induction in the photoperiods longer than the critical limit that is between 14 and 16 h at 18°C in strawberry.

FvTFL1 mediates thermal flowering response

Our gene expression analyses suggested that FvTFL1 affects not only photoperiodic flowering but also temperature-mediated flower induction in strawberry. To test the functional role of FvTFL1 and FvSOC1 in the temperature induction of flowering, we studied the thermal response in previously reported transgenic strawberry lines overexpressing *FvSOC1* and *FvTFL1* under the constitutive Cauliflower mosaic virus *35S* promoter (Koskela *et al.*, 2012; Mouhu *et al.*, 2013). We subjected two *35S:FvSOC1* and *35S:FvTFL1* lines and non-transgenic control plants to the cool temperature treatment under LD for 32 days. Control plants flowered, on average, after 47 days of the temperature treatment, whereas all plants of *35S:FvSOC1* and *35S:FvTFL1* lines remained vegetative. In another experiment, a single *35S:FvTFL1* line was grown for 6 weeks under SD and LD conditions at 10°C, but no flowering was observed (Table 2).

Gene expression analysis revealed that cool temperature treatment reduced *FvSOC1* mRNA levels already within 16 days, whereas *FvTFL1* mRNA level was decreased only at a later time point in parallel with the induction of *FvFUL1* and *FvAP1* in the shoot apices of the control plants (Figures 4 and S3). In *35S:FvSOC1* plants, however, in line with the non-flowering phenotype, cool temperature neither suppressed the expression of *FvTFL1* nor activated *FvFUL1* or *FvAP1* (Figures 4b,c and S3). In concordance with the idea that FvSOC1 functions upstream of *FvTFL1* (Mouhu *et al.*, 2013), *FvTFL1* over-expression did not change the expression of *FvSOC1*, as its expression was similarly affected by cool temperature in *35S:FvTFL1* lines

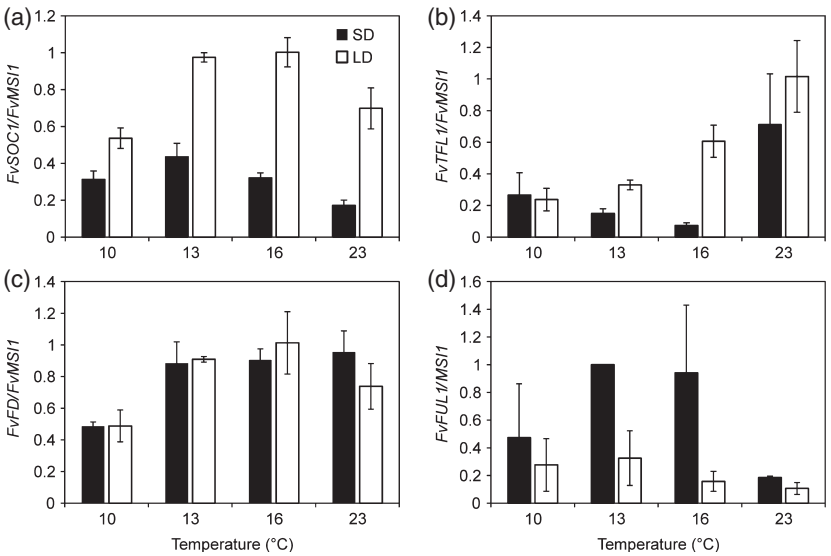

Figure 2. The expression of flowering genes in the shoot apex of strawberry plants. The plants were grown under indicated conditions for 4 weeks before sampling and the relative expression of *FvSOC1* (a), *FvTFL1* (b), *FvFD* (c) and *FvFUL1* (d) was analysed. Values are means of three biological replicates ± standard deviation.

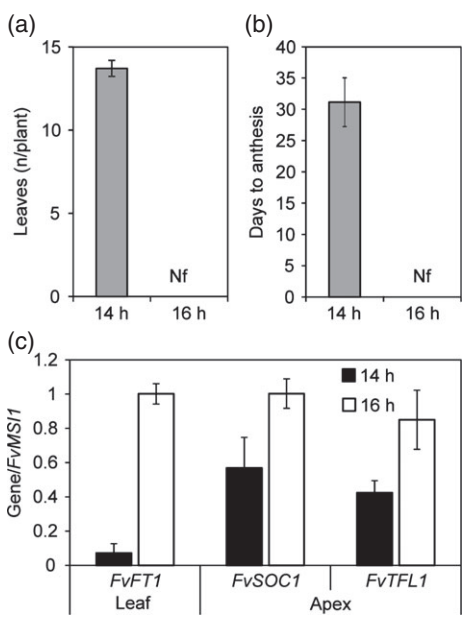

Figure 3. The effect of photoperiod on flowering time and the expression of flowering genes in strawberry. Flowering time of strawberry plants grown under 14 and 16 h photoperiods is shown as the number of leaves in the primary leaf rosette before the terminal inflorescence (a) or as days until anthesis (b). Values are means of 10 (a) or 21 (b) plants ± standard deviation (SD); Nf = no flowering. (c) The expression of *FvFT1* in the leaves and *FvTFL1* and *FvSOC1* in the shoot apex after 4 weeks of treatments. Values are means of three biological replicates ± standard deviation.

than in control plants (Figure 4a). However, the activation of the floral markers *FvFUL1* and *FvAP1* did not occur in *FvTFL1* over-expression lines (Figures 4c and S3).

To find out if the high *FvTFL1* expression level suppresses SD induction of flowering at high temperature (Figure 2b) we subjected two *FvTFL1* RNAi lines to 23°C treatment under SD and LD (Koskela *et al.*, 2012). Although

Table 2 The effect of *FvTFL1* overexpression or RNAi silencing on flowering in strawberry. The percentage of flowering plants grown under indicated temperature-photoperiod combinations is shown. The number of plants in each treatment is shown in parentheses

	10°C		23°C	
	SD	LD	SD	LD
Control	100 (7)	100 (7)	0 (5)	0 (8)
FvTFL1 RNAi #1	100 (4)	100 (4)	100 (4)	67 (6)
FvTFL1 RNAi #2	100 (6)	100 (8)	67 (6)	80 (10)
35S:FvTFL1 #1	0 (6)	0 (6)	0 (6)	0 (6)

LD, long day; SD, short day.

wild type plants stayed vegetative after these treatments, silencing of *FvTFL1* enabled photoperiod-independent flower induction in most plants (Table 2). Therefore, we suggest that the high temperature induction of *FvTFL1* sets an upper temperature limit for the flower induction in strawberry. Moreover, our finding that the over-expression of *FvTFL1* prevents cool temperature induction of flowering suggests that the suppression of *FvTFL1*, which is observed at cool temperatures, permits the photoperiod-independent flowering to occur at temperatures below 13°C.

High temperature inhibition of flowering remains in *FvSOC1* RNAi plants

To understand the role of FvSOC1 in the suppression of flowering by high temperature, we tested temperature responses of *FvSOC1* RNAi lines (Mouhu *et al.*, 2013; Figure S4a). At 16°C, the RNAi line #1 flowered at the same time in both SD and LD. Also the RNAi line #3 flowered in both photoperiods, but slightly earlier in SD, whereas wild type plants flowered only after the SD treatment (Figure 5a). In line with these results, SD suppressed *FvTFL1* in wild type plants, and reduced *FvTFL1* mRNA

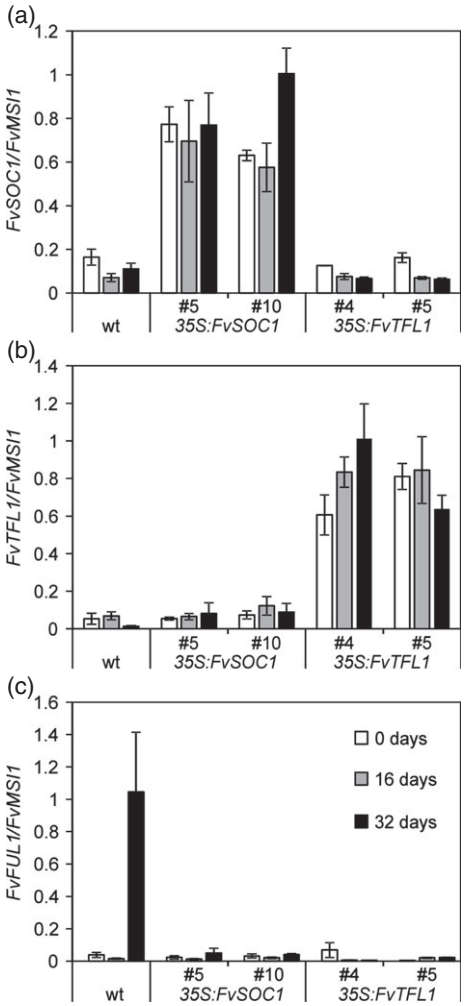

Figure 4. The expression of flowering genes in *FvSOC1* and *FvTFL1* overexpression lines.The expression of *FvSOC1* (a), *FvTFL1* (b) and *FvFUL1* (c) in the indicated transgenic lines 0, 16 or 32 days after the beginning of the cool temperature treatment (11°C, LD). Values are means of three biological replicates ± standard deviation.

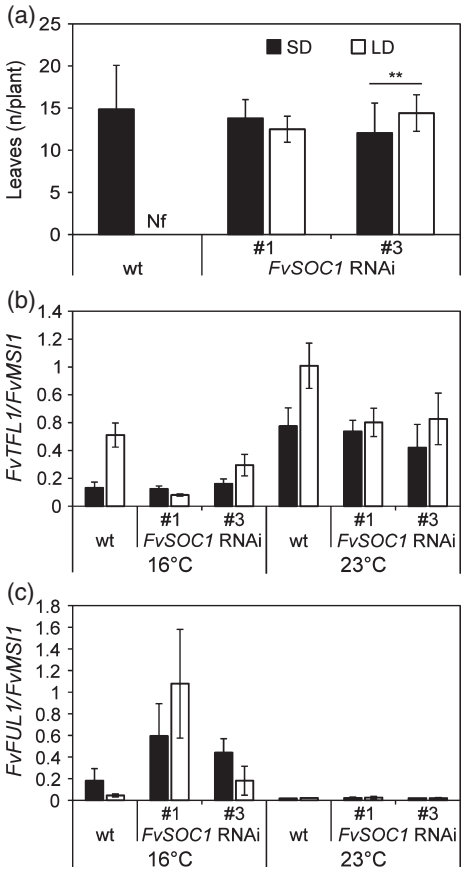

Figure 5. Flowering time and the expression of flowering genes in *FvSOC1* RNAi lines.
(a) Flowering time of *FvSOC1* RNAi lines and control plants after 6-week treatment under SD or LD at 16°C is shown. Flowering time is indicated as the number of leaves in the primary leaf rosette before the terminal inflorescence. Values are means of 18–22 plants ± standard deviation. The main effect of the genotype ($P < 0.01$) and the interaction of the genotype and the photoperiod ($P < 0.001$) were significant for flowering plants. Asterisks indicate significant difference between photoperiods according to the Tukey's test (**$\alpha = 0.01$). Control plants were not analysed by Tukey's test since they did not flower under LD. The flowering data at 23°C is not shown, since none of the plants flowered. The expression of *FvTFL1* (b) and *FvFUL1* (c) in the shoot apex samples of *FvSOC1* RNAi lines and wild type (wt) plants under different photoperiod-temperature combinations. Values are means of three biological replicates ± standard deviation.

levels were observed in both photoperiods in *FvSOC1* RNAi lines (Figure 5b). Floral marker genes *FvFUL1* and *FvAP1* were induced in parallel with the suppression of *FvTFL1* and flowering responses in both *FvSOC1* silencing lines and wild type plants (Figures 5c and S4b).

Although the silencing of *FvSOC1* caused the photoperiod-independent flowering at 16°C in this study and at 18°C in a previous work (Mouhu *et al.*, 2013), higher temperature of 23°C prevented flowering of *FvSOC1* RNAi plants similarly to the wild type in both photoperiods. At 23°C, the expression level of *FvTFL1* was higher than at 16°C especially under SD, but a weak photoperiodic effect on the expression of this gene still remained (Figure 5b). At 23°C, however, the silencing of *FvSOC1* decreased the expression of *FvTFL1* only under LD, to the same level observed in both wild type and transgenic lines under SD. Taken together, FvSOC1-dependent photoperiodic effect on *FvTFL1* mRNA level still remains at 23°C. However, our data indicate that another unknown factor highly activates *FvTFL1* at this temperature preventing the SD induction of flowering.

DISCUSSION

Leaf-expressed FT is a central signaling molecule in both photoperiodic and thermal induction of flowering in *Arabidopsis* (Blazquez *et al.*, 2003; Balasubramanian *et al.*, 2006; Corbesier *et al.*, 2007; Pin and Nilsson, 2012; Song *et al.*, 2013). Also in the perennial SD plant strawberry, FvFT1 plays a role in the photoperiodic flowering, but in contrast with *Arabidopsis FT*, the expression of *FvFT1* in

leaves correlates negatively with the flower induction so that the expression of the gene is only detected under LD. FvFT1 activates *FvSOC1* in the shoot apex under LD, but this does not lead to flowering because FvSOC1 promotes the expression of *FvTFL1*, which prevents flower induction (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014). In SD, however, these genes are suppressed and flower induction occurs. Temperature strongly affects photoperiodic responses in strawberry (Heide and Sønsteby, 2007; Heide *et al.*, 2013), and our current results underline the major role of FvTFL1 also in the thermal induction of flowering. However, distinct mechanisms may mediate photoperiod and temperature signals to adjust the expression of *FvTFL1* in the shoot apex.

TFL1 mediates thermal flowering responses

Previous studies in *F.* × *ananassa* and several British and Norwegian strawberry accessions as well as our current results in a Finnish accession showed that cool temperatures around 9–13°C induce flowering independently of the photoperiod in these species, SD is required for flower induction at intermediate temperatures, and temperatures above 19–23°C prevent the induction (Table 1; Ito and Saito, 1962; Battey *et al.*, 1998; Heide and Sønsteby, 2007; Mouhu *et al.*, 2013; Heide *et al.*, 2013). Our results on gene expression levels and transgenic lines support the idea that changes in the *FvTFL1* mRNA level are critical for setting these temperature limits for flower induction.

We show here that *FvTFL1* expression level correlates negatively with flower induction in various photoperiod–temperature combinations. Also the activation of floral marker genes *FvAP1* and *FvFUL1* (Koskela *et al.*, 2012; Mouhu *et al.*, 2013) was associated with flower induction in most cases, but not always, probably because of a too early sampling time point. According to our results, *FvTFL1* expression level is low in both SD and LD at cool temperatures of 10 and 13°C, which fully or partially replace the SD requirement of the flower induction, respectively. However, its expression gradually increases with rising temperature specifically under LD, and at 16 and 18°C, SD is obligatory for the strong suppression of *FvTFL1* and flower induction (Table 1 and Figure 2; Koskela *et al.*, 2012; Mouhu *et al.*, 2013). At 23°C, however, *FvTFL1* expression reaches high levels even under SD, and flowering is inhibited. The observed temperature response of *FvTFL1* mRNA expression likely affects thermal flowering responses, as the over-expression of *FvTFL1* prevents cool temperature induction of flowering, whereas its RNAi silencing enables flower induction to occur at high temperature (Table 2). These results indicate that, in strawberry, flower induction occurs independently of the photoperiod at cool temperatures below 13°C because of low *FvTFL1* expression level, short photoperiod is required for the suppression of *FvTFL1* to induce flowering at intermediate temperatures, whereas the strong activation of *FvTFL1* prevents flowering even under SD at temperatures above approximately 20°C.

In contrast to our findings that the role of FvTFL1 in the repression of flowering in strawberry is enhanced by increasing temperatures, TFL1 in *Arabidopsis* becomes stronger repressor at cooler temperatures (Strasser *et al.*, 2009; Hanano and Goto, 2011; Kim *et al.*, 2013). These differences are probably not caused by functional differences in the TFL1 homologues, because FvTFL1 can complement *Arabidopsis tfl1* mutant (Koskela *et al.*, 2012). However, opposite temperature responses may depend on the differences in the function of molecular pathways. In *Arabidopsis*, both FT and TFL1 can bind FD, and the increased expression of FT at higher temperatures may increase the relative proportion of flowering activating FT-FD complex at SAM leading to early flower induction (Hanano and Goto, 2011). In strawberry, however, a strong activation of *FvTFL1* by increasing temperatures seems to explain the opposite temperature response. Temperature and photoperiod may also affect the balance of different FT/TFL1-like proteins at SAM, but the role of this balance is difficult to understand, since FvFT1 may activate *FvTFL1* through FvSOC1 (Mouhu *et al.*, 2013; Rantanen *et al.*, 2014). High temperature represses flowering also in another SD plant *Chrysanthemum seticuspe* (Makino) H. Ohashi & Yonek., but the underlying molecular mechanism is different. In *C. seticuspe* the temperature signal is mediated through repression of *CsFTL3* (*FLOWERING LOCUS T LIKE3*), a *Chrysanthemum* florigen, whereas *CsTFL1* is probably not involved in this temperature response (Oda *et al.*, 2012; Nakano *et al.*, 2013). Another FT/TFL1 like protein, the anti-florigen CsAFT (ANTI-FLORIGENIC FT/TFL1 FAMILY PROTEIN) mediates the photoperiodic signal to repress flowering (Higuchi *et al.*, 2013).

FvFT1 and FvSOC1 activate *FvTFL1* according to photoperiodic signals

We have shown previously that FvFT1 and FvSOC1 mediate the LD signal to activate *FvTFL1* (Mouhu *et al.*, 2013; Rantanen *et al.*, 2014). According to data presented here, *FvFT1* shows LD specific expression at different temperatures. *FvSOC1* expression is also reduced by SD, and increasing temperature may enhance this SD response (Figures 2 and S4). However, although *FvTFL1* is gradually activated by increasing temperature in LD, the expression of *FvFT1* and *FvSOC1* does not show similar trend. This contrasts with results in *Arabidopsis* where the expression of both *FT* and *SOC1* increases along the rising temperature to advance flowering (Blazquez *et al.*, 2003; Balasubramanian *et al.*, 2006; Kumar *et al.*, 2012). Several molecular mechanisms including the eviction of the histone variant H2A.Z from nucleosomes, destabilization of the floral repressor SVP or its deactivation through binding with the alternatively spliced FLM have been assigned with the

regulation of *FT* transcription (Kumar *et al.*, 2012; Lee *et al.*, 2013; Posé *et al.*, 2013). Whether similar mechanisms could affect the transcription of *TFL1* homologues in strawberry or other species is an open question.

The lack of correlation between the expression of *FvFT1/FvSOC1* and *FvTFL1* at different temperatures suggests that the temperature affects *FvTFL1* expression independently of the photoperiodic pathway. This idea is partially supported by the data on *FvSOC1* transgenic lines. We show here that high temperature strongly activates *FvTFL1* also in *FvSOC1* RNAi plants to prevent flower induction indicating that FvSOC1 does not affect the thermal activation of *FvTFL1* between 16 and 23°C. However, FvSOC1 mediates the LD signal to promote *FvTFL1* expression at this temperature range, as the photoperiodic effect on *FvTFL1* mRNA level is attenuated in *FvSOC1* RNAi plants compared with wild type (Figure 5; Mouhu *et al.*, 2013). A major finding is that the silencing of *FvSOC1* has no effect on the strong activation of *FvTFL1* that is observed between 16 and 23°C in SD. This result supports the hypothesis that an unknown transcriptional activator prevents the induction of flowering at high temperatures by activating *FvTFL1* mRNA expression independently of FvSOC1. We suggest that this activator of *FvTFL1* sets the upper limit for the temperature range, where flower induction can take place, and below this limit, flower induction depends on FvFT1/FvSOC1-mediated photoperiodic signals (Mouhu *et al.*, 2013). A mechanistic understanding of high temperature inhibition of flowering requires the identification of this transcriptional activator, which remains elusive although the spatial regulation of *TFL1* transcription is understood at some level in *Arabidopsis* (Ratcliffe *et al.*, 1999; Liu *et al.*, 2013).

Although *FvTFL1* mRNA level is not affected by photoperiod at cool temperatures that induce flowering independently of the day length, a weak photoperiodic effect on the expression of *FvSOC1* was observed also at this temperature. In contrast, both genes are affected by the photoperiod at 13°C. These findings are in line with the idea that the photoperiodic pathway, which operates through FvSOC1 (Mouhu *et al.*, 2013; Rantanen *et al.*, 2014), activates *FvTFL1* only at temperatures above a certain limit. However, we cannot fully exclude the possibility that changes in the *FvSOC1* expression may affect *FvTFL1* mRNA levels at cool temperature, since the expression of *FvSOC1* was reduced before *FvTFL1* at 11°C (Figure 4). In addition, *35S:FvSOC1* plants retain high *FvTFL1* expression levels and are not induced to flower at cool temperature. However, this effect is possibly caused by the high ectopic expression of *FvSOC1* in these plants and does not necessarily reflect the natural role of *FvSOC1* at cool temperatures. Taken together, our data are in line with the idea that distinct photoperiodic and temperature signaling pathways can induce flowering by affecting *FvTFL1* mRNA expression. The temperature pathway dominates and sets limits of the narrow temperature range where the photoperiodic pathway is functional.

We also explored the association of gene expression levels with the critical photoperiod for flower induction and observed that the critical photoperiod is between 14 and 16 h at 18°C, in line with previous results in Norwegian strawberry accessions (Heide and Sønsteby, 2007). We found a gradual reduction of *FvFT1* expression under shortening photoperiods and reduced expression levels of *FvSOC1* and *FvTFL1* in 14 h photoperiod compared with longer day lengths, indicating that the gradual deactivation of the photoperiodic pathway sets the critical photoperiod for flower induction. At 15°C, however, Heide and Sønsteby (2007) observed longer critical day length. This result further supports the idea that the photoperiodic pathway becomes gradually unconnected with the transcriptional regulation of *FvTFL1* at decreasing temperatures.

FvTFL1 integrates photoperiod and temperature signals to inhibit flower induction

In several species of the Rosaceae family, homologues of TFL1 are strong repressors of flowering, which determine the timing of seasonal flower initiation (Kotoda *et al.*, 2006; Flachowsky *et al.*, 2012; Freiman *et al.*, 2012; Iwata *et al.*, 2012; Koskela *et al.*, 2012). Based on our previous studies (Koskela *et al.*, 2012; Mouhu *et al.*, 2013; Rantanen *et al.*, 2014) and results presented here, we propose the following model on the integration of environmental signals by *FvTFL1* (Figure 6). Cool temperatures below 13°C suppress *FvTFL1* independently of the photoperiod allowing flower induction to take place even under LD, whereas at high temperatures above approximately 20°C, an unknown transcriptional activator of *FvTFL1* prevents flowering by highly increasing *FvTFL1* mRNA levels in both SD and LD. At temperatures between these limits, however, flower induction only occurs under the photoperiods below a critical limit, after the deactivation of the *FvFT1-FvSOC1-FvTFL1* pathway.

Although many perennials show strong latitudinal clines in their photoperiodic responses (Heide, 1974; Böhlenius *et al.*, 2006), no such cline was detected in Norwegian strawberries (Heide and Sønsteby, 2007). We suggest that the observed interaction of photoperiod and temperature in the timing of flower induction in strawberry may allow the floral development to begin in correct time in autumn in different climates, and this may contribute to the wide geographical distribution of this species in the Northern hemisphere (Liston *et al.*, 2014). In current climate change scenarios, such a flexibility to respond to environmental signals would be extremely useful in perennial crops. As the interaction of photoperiod and temperature has been shown to time the annual growth cycle in many perennials (Williams, 1960; Hall and Ludwig, 1961; Junttila, 1980;

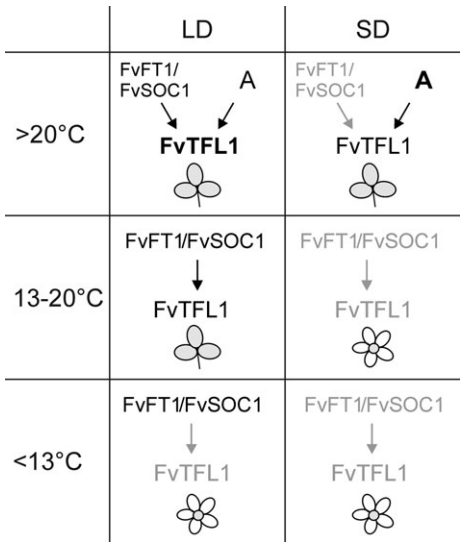

Figure 6. Model showing the interaction of photoperiodic and temperature signals in the induction of flowering in strawberry.
The genes/proteins activated by each condition are shown in black and inactive/repressed parts of the pathway are shown in grey. A = unknown transcriptional activator.

Heide, 2008; Sønsteby and Heide, 2009), it would be important to explore if similar mechanisms mediate these responses in different species.

EXPERIMENTAL PROCEDURES

Plant material

Seasonal flowering SD accession of the woodland strawberry (Accession number PI551792; National Clonal Germplasm Repository, Corvallis, USA) and previously reported *FvSOC1* and *FvTFL1* over-expression and RNAi lines were used (Koskela *et al.*, 2012; Mouhu *et al.*, 2013). Seedlings were used in all experiments that did not include transgenic lines, whereas experiments including transgenic plants were carried out using clonally propagated plants (runner cuttings). Plants were raised in a greenhouse under non-inductive photoperiod and temperature conditions (18/6 h light/dark; 22 ± 1°C). High pressure sodium (HPS) lamps (Airam 400W, Kerava, Finland, www.airam.fi) were used as a supplemental light with the intensity of 150 μmol m^{-2} sec^{-1}. Plants were grown in 8 × 8 cm pots. Fertilized peat (Kekkilä, Vantaa, Finland, www.kekkila.com) supplemented with 25% (v/v) of vermiculite (Ø2 mm) was used as a growing medium. Plants were fertilized with liquid fertilizer (Kekkilä) biweekly. Emerging runners were removed every second week. Seedlings were transferred to the treatments when they had approximately 6–8 leaves and runner propagated plants approximately 1 month after rooting.

Treatments

Plants were grown in a greenhouse under 18 h LD at 22 ± 1°C before photoperiod and temperature treatments (see details above). Photoperiod and temperature treatments were carried out in growth rooms equipped with warm white light-emitting diode (LED) lamps (Bar 604; Valoya Oy, Helsinki, Finland, www.valoya.com). The light intensity was 200 μmol m^{-2} sec^{-1}. In the growth rooms, plants were subjected to the temperatures of 10, 13, 16, and 23°C under both LD (18 h) and SD (12 h) for 6 weeks. However, *35S:FvSOC1* and *35S:FvTFL1* plants as well as their control plants were subjected to cool temperature treatment (11°C under LD) for 32 days in a greenhouse room equipped with darkening curtains. These plants were illuminated with HPS lamps 12 h daily (150 μmol m^{-2} sec^{-1}), and the photoperiod was extended for 6 h in the evening by using incandescent bulbs (10 μmol m^{-2} sec^{-1}). After treatments, plants were grown under initial growing conditions (18 h LD at 22 ± 1°C) until the anthesis.

Growth observations

Flowering time observations were carried out two times per week to record the date of first open flower. Flowering time was indicated as the number of days from the end of the treatment to the anthesis. Developmental stage of flowering was observed as the number of leaves formed in the primary leaf rosette between the beginning of the treatment and the formation of the terminal inflorescence.

Sampling, RNA extraction, cDNA synthesis and real-time polymerase chain reaction (PCR)

Plants were sampled for gene expression analysis during the treatments as indicated in the figure legends. For the leaf samples the middle leaflet of the youngest unfolded leaf, and for shoot apex samples several approximately 1 mm pieces containing SAM and youngest leaf initials were collected as three biological replicates. Shoot apex samples were collected 4 h after dawn, whereas the leaf samples were taken 16 h after dawn to match *FvFT1* expression peak (Koskela *et al.*, 2012).

RNA extraction was carried out by using pine tree method (Monte and Somerville, 2002). For cDNA synthesis (Superscript III reverse transcriptase; Invitrogen, Thermo Fischer Scientific Inc., Waltham, MA, USA, www.lifetechnologies.com), 1 μg of total RNA was used. Real-time PCR reactions were performed in LightCycler 480-instrument (Roche, Basel, Switzerland, www.lifescience.roche.com) using SYBR Green Master Mix (Roche) and 3 μM primer mix (F+R). Real-time PCR conditions were described earlier by Rantanen *et al.* (2014). Three biological and three technical replicates were analysed in each experiment. Relative expression of selected genes was calculated by $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) with stable *FvMSI1* as a normalization gene (Rantanen *et al.*, 2014). Real-time PCR primers used are listed in Table S1. Primer efficiencies were close to 2 for all primer pairs.

Statistical analyses

When appropriate, averages were subjected to analysis of variance using GLM procedure and pairwise comparisons were performed using option TUKEY in the MEANS statement in SAS statistical program (SAS/STAT software, version 9.3 of the SAS System for Windows, SAS Institute Inc. Cary, NC, USA, www.sas.com). For the non-parametric analysis, Kruskall–Wallis rank sum test [one-way analysis of variance (ANOVA)] was used. The non-parametric pairwise comparisons were calculated using Dunn's rank sum test for unequal group sizes (Zar, 1999).

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SUPPORTING INFORMATION

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

Figure S1. The expression of *FvFT1* in different photoperiods.

Figure S2. The expression of *FvAP1* in different temperature-photoperiod combinations.

Figure S3. The expression of *FvAP1* after 4 weeks of cool temperature treatment.

Figure S4. The expression of *FvSOC1* and *FvAP1* in *FvSOC1*-RNAi lines.

Table S1. qPCR primers used in this study.

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