SENSITIVE TO FREEZING6 Integrates Cellular and Environmental Inputs to the Plant Circadian Clock[1][W][OA]

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The *sensitive to freezing6* (*sfr6*) mutant of Arabidopsis (*Arabidopsis thaliana*) is late flowering in long days due to reduced expression of components in the photoperiodic flowering pathway in long-day photoperiods. Microarray analysis of gene expression showed that a circadian clock-associated motif, the evening element, was overrepresented in promoters of genes down-regulated in *sfr6* plants. Analysis of leaf movement rhythms found *sfr6* plants showed a sucrose (Suc)-dependent long period phenotype; unlike wild-type Arabidopsis, the clock in *sfr6* plants did not have a shorter rhythm in the presence of Suc. Other developmental responses to Suc were unaltered in *sfr6* plants, suggesting insensitivity to Suc is restricted to the clock. We investigated the effect of *sfr6* and Suc upon clock gene expression over 24 h. The *sfr6* mutation resulted in reduced expression of the clock components *CIRCADIAN CLOCK ASSOCIATED1*, *GIGANTEA*, and *TIMING OF CAB1*. These changes occurred independently of Suc supplementation. Wild-type plants showed small increases in clock gene expression in the presence of Suc; this response to Suc was reduced in *sfr6* plants. This study shows that large changes in level and timing of clock gene expression may have little effect upon clock outputs. Moreover, although Suc influences the period and accuracy of the Arabidopsis clock, it results in relatively minor changes in clock gene expression.

Plants experience many fluctuations in their environment, but the daily change between dark and light conditions is both predictable and extremely significant in terms of the direct effect it has on photosynthesis. Green plants need to tune their metabolism and biochemistry to take account of times when photosynthesis is or is not possible. Many of the changes that occur throughout the diurnal cycle are controlled at the transcript level, such that 30% to 50% of genes in the model plant Arabidopsis (*Arabidopsis thaliana*) are rhythmically expressed in light:dark conditions (Blasing et al., 2005). A core of about 6% of genes continues to be rhythmic under constant light conditions (Harmer et al., 2000), indicating that these genes are under the direct control of the circadian clock. The clock controls the timing of key areas of plant metabolism, including the synthesis and degradation of starch and other carbohydrates (Harmer et al., 2000), allowing plants to optimize their metabolism to suit daily changes in the environment. The Arabidopsis clock is made up of a series of interlocked transcription-translation feedback loops (Locke et al., 2005, 2006; Zeilinger et al., 2006) centered on the highly homologous MYB transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY; Schaffer et al., 1998; Wang and Tobin, 1998; Mizoguchi et al., 2002). These proteins, together with their transcripts, have peak expression around dawn. They drive the expression of other clock components such as TIMING OF CAB1 (TOC1), whose transcript is maximally expressed in the evening (Strayer et al., 2000; Alabadi et al., 2001), and its homologues, the *PSEUDORESPONSE REGULATOR* family, which are expressed in sequential waves throughout the day (Matsushika et al., 2000; Makino et al., 2002). CCA1 and LHY repress *TOC1* transcription (Alabadi et al., 2001; Perales and Mas, 2007), whereas the level of TOC1 is regulated by ZEITLUPE (ZTL; Somers et al., 2000). ZTL targets TOC1 protein for degradation via the proteasome, a process that is dark dependent (Mas et al., 2003b).

The clock receives inputs from the external environment from several types of photoreceptors, including phytochromes and cryptochromes (for review, see Millar, 2003; Larner et al., 2005), which allow it to entrain to the light:dark cycles of day and night. Control of target genes regulated by CCA1 and LHY is via cis-elements such as the evening element (EE; Harmer et al., 2000) or the CCA1-binding site (Wang et al., 1997; Michael and McClung, 2003) in their promoters. The EE (AAAATATCT) was identified as

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a motif found in a number of genes under circadian regulation that exhibited peak expression at the end of the day (Harmer et al., 2000). The EE in the *TOC1* promoter is directly bound by CCA1 (Perales and Mas, 2007). EEs are also found in the promoters of *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*FKF1*; a homolog of *ZTL*) and *GIGANTEA* (*GI*; Imaizumi et al., 2003).

The clock has a large influence on flowering time via the photoperiodic flowering pathway (Imaizumi and Kay, 2006). Arabidopsis is a facultative long-day plant, flowering earlier and with fewer leaves in long days than in short ones. FKF1 acts as a photoreceptor in the photoperiodic control of flowering (Nelson et al., 2000; Imaizumi et al., 2003). FKF1 indirectly regulates the level of *CONSTANS* (*CO*; Imaizumi et al., 2005); CO in turn promotes flowering (Putterill et al., 1995) by activating FLOWERING LOCUS T (FT; An et al., 2004). *GI* acts both in the photoperiodic pathway and in the clock (Fowler et al., 1999; Mizoguchi et al., 2005; Martin-Tryon et al., 2007).

While much is known about the plant clock's responses to light and temperature (Gould et al., 2006), less is known about how it responds to other changes in the environment. Somewhat surprisingly, the major factor controlling the enormous daily variation in gene expression in diurnal conditions is not the clock but sugar, the availability of which varies across the day and night (Blasing et al., 2005). This means that the pattern of gene expression in Suc-grown plants is vastly different from those without Suc, even if the light and temperature regime is otherwise identical. Because Suc enhances the brightness of *LUCIFERASE* (*LUC*) bioluminescence (developed as a reporter of circadian gene activity; Millar et al., 1995), studies on the Arabidopsis circadian clock are most often carried out using plants grown on media to which supplementary Suc has been added, even when *LUC* reporter activity is not recorded (Harmer et al., 2000; Edwards et al., 2005). In contrast, flowering time experiments are usually conducted on plants grown on soil without supplementary Suc. Given the role of the circadian clock in regulating the photoperiodic flowering pathway, this difference in experimental protocol has the potential for confounding experimental analysis and means that a systematic investigation of the effect of Suc on the major clock components and circadian outputs is timely.

During the course of routine plant growth, we observed that the Arabidopsis *sensitive to freezing6* (*sfr6*) mutant (Knight et al., 1999) phenocopied some circadian clock mutants, in that it was late flowering. Further investigation revealed wild-type Arabidopsis had a clock that was sensitive to Suc, producing a shorter period when plants were grown with supplementary Suc in the same light intensity. This change was not seen in *sfr6* plants; hence, SFR6 is required for the clock to respond to Suc. Analysis revealed reduced expression of components of the clock and flowering time pathways in *sfr6* plants. Suc supplementation altered the amplitude and/or phase of clock-associated gene expression in wild-type plants, but these differences were reduced in or absent from *sfr6*.

RESULTS

sfr6 Is Late Flowering

We observed that *sfr6* plants flowered significantly later than wild type in long days (LD 16:8), having on average (under our conditions) 24 rosette leaves at the time of flowering compared with only 15 in wild-type plants ($P < 0.0001$, one-tailed t test; Fig. 1A). A significant difference in the age of plants at the time of flowering was also recorded (Supplemental Fig. S1; data shown in Fig. 1A and Supplemental Fig. S1 represent 22 plants per genotype).

To examine the cause of late flowering in *sfr6*, we measured expression of genes known to be involved in the control of flowering in Arabidopsis (Fig. 1, B and

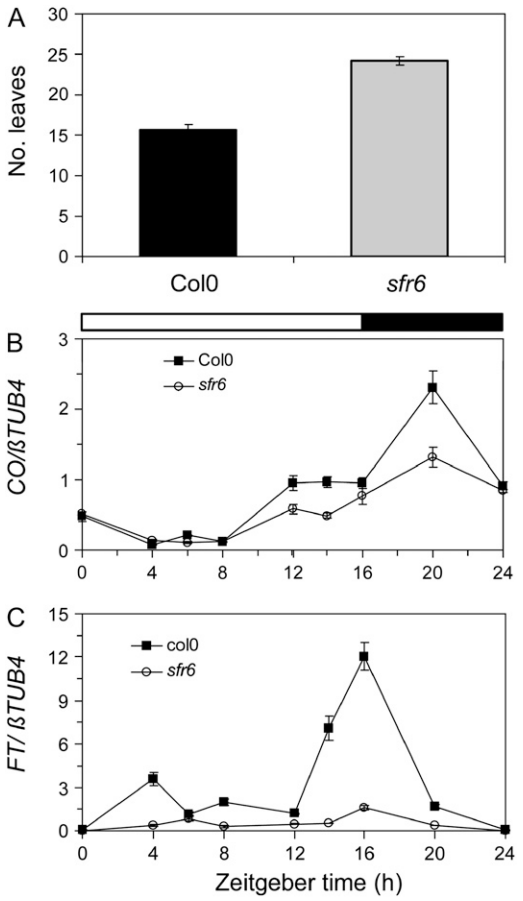

Figure 1. *sfr6* is a late flowering Arabidopsis mutant. A, Flowering time. Mean (±SEM) number of leaves at flowering in Col0 and *sfr6* plants in long-day photoperiods (LD 16:8). B, *CO* expression in LD 16:8 1× MS medium. C, *FT* expression in LD 16:8 1× MS medium. White bar, Light on (day; zeitgeber time [ZT] 0–16); black bar, light off (night; ZT 16–24). Each value is the mean of three separate quantitative RT-PCRs normalized to contemporaneous expression of the *βTUB4* control (mean ± SD).

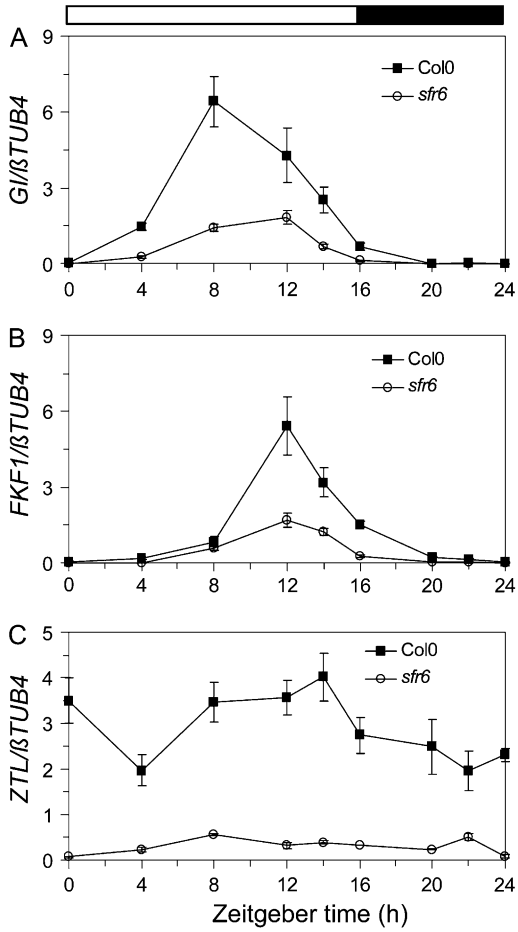

Figure 2. Effect of *sfr6* mutation on genes affecting flowering. A, *GI* expression in LD 16:8 1× MS medium. B, *FKF1* expression in LD 16:8 1× MS medium. C, *ZTL* expression in LD 16:8 1× MS medium. White and black bars indicate day and night, respectively, as in Figure 1, B and C. Each value is the mean of three separate quantitative RT-PCRs normalized to contemporaneous expression of the *βTUB4* control (mean ± SD).

C). When we examined *CO* expression in seedlings in LD 16:8 photoperiods, we found that expression of *CO* was reduced during the late day and night in *sfr6* (Fig. 1B). Moreover, expression of *FT* was severely reduced in *sfr6* seedlings in long days (Fig. 1C). As *CO* is involved in photoperiodic time measurement and *FT* levels determine the timing of the floral transition, reduction in expression of these genes explains the late flowering of *sfr6* plants.

We also examined expression of genes that act upstream of *CO* in the photoperiodic pathway regulating flowering time. The *gi* mutant resembles *sfr6*, as it is also late flowering in long days (Fowler et al., 1999). In *sfr6* plants grown in LD 16:8 photoperiods, expression of *GI* was reduced and the peak of expression delayed compared with wild-type seedlings (Fig. 2A). FKF1 is a blue light photoreceptor regulating the photoperiodic flowering time pathway. As *fkf1* plants are late flowering in long days and, like *sfr6*, have reduced levels of *CO* and *FT* (Imaizumi et al., 2003), we examined *FKF1* expression in *sfr6*, finding it was also reduced in *sfr6* (Fig. 2B). *ZTL* is a homolog of *FKF1*, and in long days the *ztl-1* mutant produced 50% more leaves at flowering than wild type and took a longer time to flower (Somers et al., 2000). We found that the level of *ZTL* was greatly reduced in *sfr6* (Fig. 2C).

Clock-Controlled Genes Are Down-Regulated in *sfr6*

Having observed an effect on the pathway leading to control of flowering time, we sought to discover whether the circadian clock also was affected in *sfr6*. Microarray analysis was used to compare global gene expression in wild-type and *sfr6* seedlings grown in LD 16:8 photoperiods during the light period. When we analyzed data from a full genome chip, we found 209 genes were least 2× down-regulated in *sfr6* (Supplemental Table S1). Analysis of 500 bp of upstream sequence from these genes by the Regulatory Sequence Analysis Tools (van Helden, 2003) Pattern Matching Function revealed TATAAAATATCTT as the most significant consensus sequence. This was of particular interest to us, as this motif contains the EE (underlined), suggesting that the *sfr6* mutation down-regulates expression of circadian clock-controlled genes.

sfr6 Plants Show a Suc-Dependent Circadian Phenotype

The overrepresentation of the EE among genes down-regulated in *sfr6* coupled with the knowledge that many flowering time mutants also have defects in their circadian clocks led us to investigate whether *sfr6* plants had altered circadian function. We tested this possibility by examining leaf movement rhythms in *sfr6* and wild-type seedlings. As the leaf movement rhythm is controlled by the circadian clock, determination of its frequency in constant light provides a measure of the endogenous, free-running period of the plant. Leaf movement experiments are commonly performed using plants grown with supplemental Suc, but, as our flowering time and microarray experiments had been conducted on plants grown without Suc, we wanted to observe clock behavior in plants growing under similar conditions. We therefore measured leaf movement rhythms in wild-type and *sfr6* plants grown without supplementary Suc. There was no difference in period length between wild-type and *sfr6* seedlings under these conditions (ecotype Columbia [Col0] period = 25.12 h versus *sfr6* period = 25.15 h; one-tailed *t* test, $t = -0.06$, not significant [ns]; Fig. 3A; breakdown of both experiments is in Table I).

To allow comparison with the majority of previously published leaf movement data, we repeated the leaf movement recordings using plants grown on media supplemented with 3% (w/v) Suc. Analysis of rhythms showed that under these conditions, *sfr6* plants had a significantly longer period than wild type (Col0 period = 24.44 h versus *sfr6* period = 25.24 h; one-tailed *t* test, $t = -4.28$, $P < 0.001$; Fig. 3D;

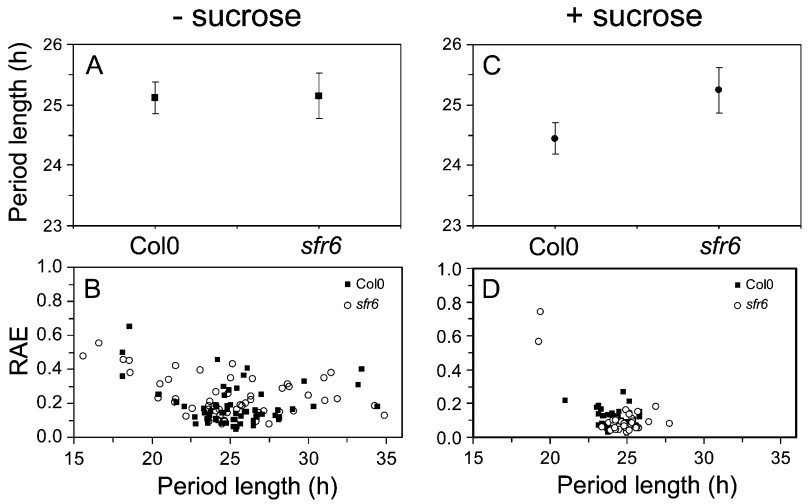

Figure 3. Effect of *sfr6* mutation and Suc on the Arabidopsis clock. Period lengths assayed from leaf movement rhythms in LL after LD 12:12 on 1× MS medium or 1× MS medium with 3% Suc. Analysis of leaf movement rhythms in plants grown without (A and B) or with (C and D) supplementary Suc. A, Leaf movement rhythms of Col0 and *sfr6* seedlings grown without Suc. Values shown are means and SEM of two independent experiments. B, RAE scores for seedlings grown without Suc. Each symbol shows the RAE and period length of an individual seedling. Data from one experiment shown; two independent replicates gave similar results. C, Leaf movement rhythms of Col0 and *sfr6* seedlings grown with 3% Suc. Values shown are means and SEM of two independent experiments. D, RAE scores for leaf movement periods of seedlings grown with Suc. Each symbol shows the RAE and period length of an individual seedling (data from one experiment shown; two independent replicates gave similar results).

breakdown of all experiments is in Table I). This shows that the *sfr6* mutation caused a conditional circadian phenotype that resulted in a leaf movement rhythm longer than wild type but only in the presence of Suc.

It appears that this difference occurs because the free-running period of the wild-type Arabidopsis clock is sensitive to Suc, but *sfr6* is unaffected by the presence of Suc (Fig. 3, A and C); analysis of the leaf movement data found wild-type plants had shorter clock periods when they were grown with Suc (mean period for all Col0 seedlings on 1× Murashige and Skoog [MS] 25.1 h versus mean period on 1× MS with 3% Suc 24.4 h; $t = -2.43$, $P < 0.01$, one-tailed t test), but *sfr6* plants did not (mean period for all *sfr6* seedlings on 1× MS 25.1 h versus mean period on 1× MS with 3% Suc 25.2 h; $t = 0.24$, ns, one-tailed t test).

We noticed that each genotype returned a very broad range of period values when grown without Suc (Fig. 3, A and B), but values were much more tightly clustered around the mean when plants were grown with Suc (Fig. 3, C and D). As both genotypes showed similar behavior in this respect, this is unlikely to result from the *sfr6* mutation. The light intensity, photoperiod, and temperature were all equivalent in both sets of experiments; hence, this result may indicate that Suc acts to facilitate entrainment to the light:dark cycle.

To determine the robustness of leaf movement rhythms, we determined the relative amplitude error (RAE) for each genotype grown with and without Suc (Johnson and Frasier, 1985; Straume et al., 1991; Plautz et al., 1997). The RAE is a measure of the sustainability and precision of rhythms; RAE values < 0.6 are considered to represent robust rhythms. RAE scores of <0.6 for individual seedlings of *sfr6* grown without Suc indicated that they retained robust rhythmicity, as

Table I. *Estimates of mean period values obtained by leaf movement analysis of Col0 and sfr6 seedlings grown on 1× MS media without (ms0) or with supplementary Suc (ms3)*

Pooled data are the mean values for all seedlings in a group as shown in Figure 3A. One-tailed *t* tests were used to determine whether to reject the null hypothesis that *sfr6* free-running period did not differ from that of Col0. a, b, and c are independent repeats of the experiments.

Experiment	Col0 Period	No. Col0 Seedlings	*sfr6* Period	No. *sfr6* Seedlings	*t*	*P*
	h		*h*			
ms0 a	25.26	63	25.07	54	0.29	0.39
ms0 b	24.99	69	25.25	44	−0.42	0.34
ms3 a	24.06	64	24.67	46	−2.70	<0.01
ms3 b	24.74	63	25.33	35	−1.94	<0.05
ms3 c	24.57	45	25.84	39	−2.89	<0.01
Pooled ms0	25.12	132	25.15	98	−0.06	0.47
Pooled ms3	24.44	172	25.24	120	−4.28	<0.001

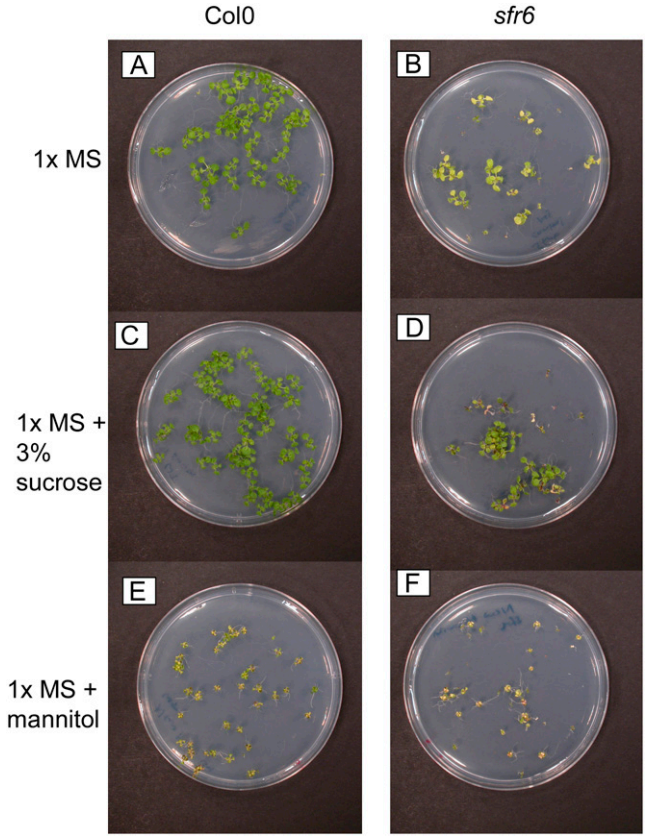

Figure 4. Wild-type and *sfr6* seedlings develop normally on 3% Suc, but an osmotically equivalent concentration of mannitol inhibits growth. Col0 (A, C, and E) and *sfr6* (B, D, and F) seedlings grown in LD 12:12 at 20°C are shown. A and B, Plants grown on 1× MS medium without supplementation. C and D, Plants grown on 1× MS medium supplemented with 3% Suc (w/v). E and F, Plants grown on 1× MS medium with mannitol (96 mM).

did wild-type seedlings (Fig. 3B). When Suc was present in the growth medium, leaf movement rhythms of individual *sfr6* seedlings again had low RAE, with a distribution that did not differ from wild type (Fig. 3D). This means that robust rhythmicity of leaf movement was retained in *sfr6* under both sets of experimental conditions. Therefore, although the free-running clock of *sfr6* takes longer to complete a cycle in the presence of Suc, this mutant retains a circadian system with many of the characteristics seen in wild-type plants.

The Effect of Suc upon the Clock Is Not a Result of Osmotic Stress

We did not observe an abnormal appearance of seedlings grown with supplementary Suc (Fig. 4, A–D). However, this does not rule out the possibility that the effect of Suc on clock function was due to osmotic stress imposed by Suc supplementation. Therefore, as a chemical control for the higher osmotic strength of MS medium containing Suc, we compared growth of wild-type and *sfr6* seedlings on plates containing 1× MS medium supplemented by 3% Suc or mannitol at isoosmolar concentrations (concentrations of equivalent osmotic strength). Both genotypes grown on mannitol were stunted and appeared yellow when compared with plants grown on Suc (Fig. 4, E and F; Supplemental Fig. S2).

This could have indicated either that mannitol exerted a negative effect on Arabidopsis seedlings that could not be attributed to its osmotic strength alone or that Suc does not in fact impose the level of osmotic stress on Arabidopsis that is caused by an isoosmolar concentration of mannitol. To distinguish between these possibilities and to test whether this was an effect specific to mannitol, plants were grown on 1× MS medium containing 3-*O*-methyl Glc (3-OMG); 3-OMG is a Glc analog that is not recognized by plants as a metabolizable sugar (Cortes et al., 2003). Like Suc, 3-OMG can be taken up by plant cells. Plants grown on a concentration of 3-OMG equivalent to the osmotic strength of 3% Suc were also stunted, similar to those grown on mannitol (Supplemental Fig. S2). Finally, we calculated the osmolarity of MS medium and grew plants on 2.9× MS medium, which has an osmolarity equivalent to that of 1× MS plus 3% Suc. Again, we observed stunted plants, though less severely stunted than those grown on 3-OMG or mannitol (Supplemental Fig. S2).

These observations led to the conclusion that the Suc in our circadian experiments was not likely to impose a major osmotic stress. Suc-grown plants appeared healthy and unlike plants grown on two alternative sugars at equivalent osmolarity or on a medium with a similar osmolarity due to an increased salt concentration. Thus, we concluded that there was no appropriate chemical osmotic control for Suc in such experiments; therefore, MS medium minus Suc was the most appropriate control treatment in these and subsequent experiments. Plants grown on these three alternative media were all developmentally and physiologically quite different to those grown on Suc or non-Suc medium and therefore would not have been appropriate control plants for our experiments. The lack of osmotic stress imposed by Suc is likely to be due to the very fact it is metabolized and therefore does not remain in the media at the concentration initially added to the plates.

Other Responses to Suc Are Not Affected by the *sfr6* Mutation

We sought to determine if the lack of effect of Suc on the clock was due to insensitivity to Suc of *sfr6* plants. Suc is known to delay germination in wild-type plants, and at high concentrations, Glc has been shown to block it, but these effects are reduced or absent in sugar-insensitive mutants such as hexokinase mutants (Jang et al., 1997). Germination rates of wild-type and *sfr6* seedlings were measured in constant darkness (DD). Germination success rates of *sfr6* were equivalent to wild type (Supplemental Table S2) on 1× MS medium and medium supplemented with Suc.

A delay in germination in response to Suc is observed in wild-type seeds (Dekkers et al., 2004). Suc delayed germination of each genotype by about 24 h (Supplemental Table S2), but the effect on *sfr6* did not differ from wild type. These results indicate the mutation does not affect germination success or timing; moreover, Suc neither promotes nor inhibits germination of *sfr6* over and above the expected response of wild-type plants.

We measured hypocotyl elongation as a further test of sensitivity to Suc, which has been shown to inhibit hypocotyl growth (Jang et al., 1997). This also tested whether *sfr6* showed defective early growth. Comparison of hypocotyl lengths of *sfr6* and wild-type seedlings growing without Suc in constant white light (LL) determined that the genotypes did not differ significantly from each other (one-tailed *t* tests, ns); this was also true of seedlings grown under similar conditions but with supplemental Suc (Table II). However, in LL, hypocotyls of both genotypes were shorter when Suc was present (Table II); this is consistent with earlier reports that Suc inhibits hypocotyl elongation (Dijkwel et al., 1997). Suc did not affect hypocotyl length of *sfr6* seedlings growing in the dark (Table II), nor did *sfr6* seedlings differ in length from wild type. Taken together, these experiments indicated that *sfr6* is not globally insensitive to sugars and that the effects we observe may be specific to the clock.

SFR6 and Suc Both Modulate Circadian Gene Expression

To investigate how the *sfr6* mutation was interacting with Suc to cause the effects on the clock, we used real-time quantitative PCR to measure level and timing of transcript accumulation for the clock components *CCA1*, *TOC1*, and *GI*. Expression of these genes was measured in seedlings of both genotypes grown with or without supplementary Suc (Fig. 5). This experiment was designed to replicate the free-running conditions of the leaf movement experiments. For this reason, seedlings were entrained in LD 12:12 photoperiods and then allowed to free-run in LL for 24 h before sampling began. Samples were collected at 3-h intervals during the second day in LL (subjective day, 24–36 h; subjective night, 36–48 h) to avoid after-effects of entrainment to the light:dark cycle.

CCA1

We examined expression of the central clock component *CCA1* in wild-type and *sfr6* plants to observe the effects of the mutation and Suc supplementation on expression of a morning clock component. In both genotypes, *CCA1* expression was rhythmic with peak levels occurring, as expected, in the morning shortly after subjective dawn; however, the amplitude of the *CCA1* rhythm was reduced in *sfr6* seedlings. The expression maximum was much lower than in wild-type regardless of whether Suc supplementation was provided (Fig. 5, A and B). Thus, the *sfr6* mutation leads to a reduction in peak *CCA1* expression.

Our results indicate Suc caused small but significant changes in *CCA1* expression in wild type. In samples collected at and before subjective dawn, *CCA1* expression in wild-type plants was higher when plants were grown with Suc (compare expression in Col0 at 24 and 48 h; Fig. 5, A and B). Expression of *CCA1* in *sfr6* seedlings did not change in the presence of Suc; *CCA1* expression in *sfr6* plants was not increased by Suc at dawn or any other time. Hence, SFR6 is needed for the Suc enhancement of the increase in *CCA1* levels seen at dawn. Examination of *CCA1* expression in *sfr6* in LD 16:8 photoperiods showed that it remained rhythmic, but, as in free-running conditions, the amplitude of the rhythm was reduced relative to that in wild-type plants (Supplemental Fig. S3a).

TOC1

We measured expression of *TOC1*, a clock component expressed during the evening and early night, to determine if the evening arm of the clock was affected

Table II. *Suc inhibits hypocotyl elongation in LL in sfr6 and wild-type plants*

Table shows mean hypocotyl lengths of wild-type and *sfr6* seedlings grown on 1 × MS medium with (ms3) or without supplementary Suc (ms0) in LL or DD. Two different seed batches for each genotype were scored separately. Hypocotyl lengths were measured after 7 d of growth using ImageJ software (www.rsb.info.nih.gov/ij/). One-tailed *t* tests were used to determine whether to reject the null hypotheses that Suc did not affect hypocotyl length (results in table) and that *sfr6* mutants did not differ from wild type. Hypocotyl lengths of *sfr6* and wild-type seedlings were equivalent in LL or DD on ms0 or ms3 (all *t* test results ns; not shown).

Genotype/Light Level	Length on ms0	ms0 SEM	*n* ms0	Length on ms3	ms3 SEM	*n* ms3	*t*	*P*
	mm			*mm*				
Col0 no. 1 LL	1.74	0.06	29	1.32	0.06	20	5.29	1.63E-06
Col0 no. 2 LL	1.63	0.06	25	1.31	0.06	18	3.78	0.0003
sfr6 no. 1 LL	1.79	0.06	25	1.43	0.06	23	4.38	3.43E-05
sfr6 no. 2 LL	1.96	0.09	26	1.60	0.07	24	3.34	0.0009
Col0 no. 1 DD	13.40	0.34	26	15.33	0.54	27	−3.01	0.002
Col0 no. 2 DD	14.90	0.28	28	14.19	0.44	27	1.36	0.09
sfr6 no. 1 DD	14.19	0.58	29	14.75	0.53	25	−0.71	0.24
sfr6 no. 2 DD	14.38	0.30	33	15.07	0.41	24	−1.34	0.09

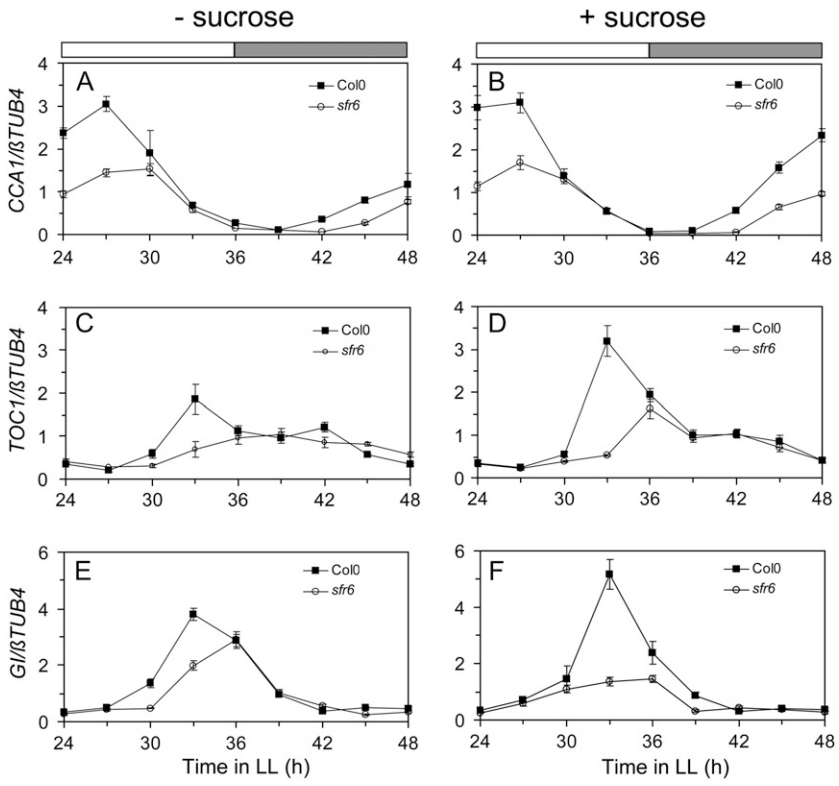

Figure 5. Effect of *sfr6* mutation and Suc on expression of clock components. Circadian clock gene expression in plants free-running on 1× MS medium (A, C, and E) or 1× MS medium supplemented with 3% Suc (w/v; B, D, and F) is shown. A and B, *CCA1* expression. C and D, *TOC1* expression. E and F, *GI* expression. To match the conditions of the leaf movement experiment, gene expression studies were conducted in LL after LD 12:12. Each value is the mean of three separate quantitative RT-PCRs normalized to contemporaneous expression of the *βTUB4* control. White bars, Subjective day (time 24–36 h); gray bars, subjective night (time 36–48 h). Means ± SD.

by the *sfr6* mutation. As CCA1 negatively regulates *TOC1*, changes in *CCA1* expression should be reflected in *TOC1* profiles. The main peak of *TOC1* expression in wild-type seedlings occurred in the late subjective day at time 33 h (Fig. 5, C and D). Both the peak of *TOC1* expression and the amplitude of the *TOC1* rhythm were reduced in *sfr6* seedlings; in addition, the time of peak expression was delayed in *sfr6* relative to wild type (Fig. 5, C and D). Consistent with this result, *TOC1* expression in *sfr6* was reduced in amplitude and rhythmicity in long-day photoperiods (Supplemental Fig. S3b).

Suc supplementation increased the amplitude of rhythmicity of *TOC1* expression in free-running wild-type plants (Fig. 5, C and D). It also produced an overall increase in *TOC1* expression; total *TOC1* expression across all time points in Col0 grown with Suc was 133% of that found in plants grown on nonsupplemented media. In contrast, although Suc strengthened the amplitude of *TOC1* rhythm in *sfr6*, it barely affected the level of expression in *sfr6*; *TOC1* expression of Suc-grown *sfr6* plants was 103% of the expression seen in plants grown without Suc (Fig. 5, C and D). Therefore, the increase in *TOC1* expression in response to Suc supplementation may require SFR6.

Expression of both *CCA1* and *TOC1* was also low relative to wild type in *sfr6* plants maintained in long-day photoperiods (Supplemental Fig. S3), showing that the reduced expression of clock components is an integral part of the *sfr6* phenotype rather than being caused by a rapid damping of rhythmicity in the mutant following release from an entraining photoperiod.

GI

It has been demonstrated previously that TOC1 is activated by GI (*TOC1* expression is reduced in the *gi;cca1;lhy* triple mutant relative to the *cca1;lhy* mutant; Locke et al., 2006). We had already observed that expression of *GI* was reduced in *sfr6* seedlings in long days (Fig. 2A). In addition to its role in regulating flowering time, *GI* acts in the circadian pathway controlling response to light:dark cycles (Martin-Tryon et al., 2007). We therefore measured *GI* expression in free-running conditions to determine whether it would respond to Suc in the growth medium. In constant light, *GI* expression in wild-type seedlings was rhythmic with a peak at 33 h in constant light (toward the end of the subjective day; Fig. 5, E and F). Consistent with the results obtained from plants entrained to an LD 16:8 photoperiodic cycle (Fig. 2A), the time of maximum expression of *GI* was delayed in *sfr6* seedlings, occurring after 36 h in LL, and its level was reduced (Fig. 5, E and F). This reinforced the earlier conclusion that SFR6 is required for correct amplitude and timing of *GI* expression and implies the mutation's effects upon *TOC1* may be a downstream consequence of altered *GI* profile.

Unlike its effects on *CCA1* and *TOC1* transcript levels, the direction of effect of Suc on *GI* expression

varied with genotype; the height of the *GI* peak in LL was increased when Suc was present in the growth media in wild type but reduced in *sfr6* (compare expression in Fig. 5, E and F). This implies that *GI* is directly sensitive to Suc and this response requires SFR6. In contrast to this finding, a recent study has suggested that *GI* expression does not respond to Suc (Usadel et al., 2008); however, this experiment used carbon-starved plants so may not be directly comparable to these experiments where plants were maintained on Suc-supplemented media throughout growth.

DISCUSSION

SFR6 Regulates the Photoperiodic Pathway and Clock Gene Expression

We have shown that, like other late flowering mutants, *sfr6* shows reduced expression of genes active in the photoperiodic pathway. The reduction and or delay in expression of *GI*, *FKF1*, *CO*, *ZTL*, and *FT* support the conclusion that *sfr6* plants have reduced sensitivity to daylength, because SFR6 is required for activation of the photoperiodic flowering pathway.

A notable aspect of the *sfr6* phenotype is the simultaneous reduction in expression of morning and evening clock components. Taken together, our results revealed an interesting contradiction. Analysis of clock component expression showed that, consistent with its effects upon the photoperiodic pathway, *sfr6* caused an overall decrease in level of clock gene expression in free-running plants, regardless of Suc supplementation. However, *sfr6* exhibited a clock phenotype differing from wild type only when plants were grown with Suc. Therefore, the large changes in clock gene expression apparent in *sfr6* were not in themselves sufficient to alter clock behavior; when grown without Suc, *sfr6* and wild-type plants had equivalent free-running periods of leaf movement despite major differences in clock gene expression between genotypes. This is an important result, as the *sfr6* mutation appears to separate expression of clock components from clock function.

Suc Acts as a Regulator of Clock Function and Interacts with SFR6

Clock gene expression was less responsive to Suc in *sfr6* than in wild type; however, this reduced response appears to be restricted to clock pathways only. *sfr6* showed normal wild-type behavior on Suc in terms of inhibition of hypocotyl elongation, germination timing, and success, suggesting that *sfr6* is not globally insensitive to Suc but that Suc-responsive pathways leading to clock function specifically are regulated by SFR6. The changes in clock gene expression caused by Suc in wild type may be responsible for the shortened period observed; however, given that the larger differences in *sfr6* and wild-type clock transcript levels measured cause no change in clock function, alternative explanations remain a possibility. Although Suc does cause moderate changes in clock gene transcript levels, it is likely that it exerts its major influence on circadian function downstream (or independently) of clock gene transcript regulation.

This study reveals the sensitivity of the Arabidopsis circadian clock to Suc. The significance of Suc in regulating the period of the circadian clock has not been recognized until now, with experiments routinely performed on plants grown on media containing 3% Suc (Harmer et al., 2000; Edwards et al., 2005; Kim et al., 2007). Our observation of reduced variability in period lengths in Suc-supplemented plants suggests that the level of sugar is an important factor involved in coordinating the plant clock with its environment. As photosynthesis takes place only during the light, the peak of *CCA1* expression normally coincides with the onset of photosynthesis and the production of sugars. The availability of experimentally applied excess Suc may cause the plant to behave as if ''dawn'' has come early by up-regulating *CCA1* during the (subjective) night. Similarly, the changed pattern of *TOC1* seen in wild-type plants grown with Suc suggests that falling Suc levels after dusk, when photosynthesis ceases, may also be a cue that resets the clock at the end of the day. Suc interference with dawn-dusk signaling in this way may be the cause of the shorter period we observe in wild type grown on supplementary Suc. In nature, an increase in Suc level may be interpreted at a cellular level by plants as an increase in light levels (which causes an increase in photosynthetic activity and hence of sugars) and so results in a shortening of the circadian period, in analogy with Aschoff's rule (day-active organisms have shorter circadian periods in brighter light; Aschoff, 1979). Our data suggest SFR6 is a component in this response to Suc.

Role of SFR6 in the Plant Circadian Clock

As the three transcripts *CCA1*, *GI*, and *TOC1* peak at different times relative to the environmental cycle, the similar effects of the *sfr6* mutation upon them suggest that SFR6 acts upstream of the clock. In *sfr6*, transcript levels of the evening genes *GI* and *TOC1* are reduced and their peaks delayed, suggesting that SFR6 acts at night. Our results imply that the effects of SFR6 and Suc may not be mediated solely via the *CCA1-TOC1-GI* feedback loops, and that, as might be expected, additional factors contribute to integrate the clock with other signals.

According to the current model of the Arabidopsis clock (Locke et al., 2005, 2006), a reduction in *CCA1* level is predicted to cause an increase in *TOC1* as CCA1 directly represses *TOC1* expression (Perales and Mas, 2007), while, in turn, TOC1 (indirectly) promotes expression of *CCA1* (Mas et al., 2003a). This feedback loop is central to current understanding of the plant clock. However, we could not replicate the patterns of

gene expression seen in *sfr6* via reductions in the core clock components using the published model (Locke et al., 2005, 2006; data not shown). The effect of *sfr6* and Suc upon *GI* expression coupled with the observation that changing the model parameters to mimic a reduction in *GI* produced the best simulation of *sfr6* suggests that determining if there is an interaction between GI and SFR6 would be a promising line of inquiry. Integration of *sfr6* into the clock model will extend our understanding of clock regulation, in particular the interactions of daily changes in cellular physiology resulting from photosynthesis with the transcription-translation feedback loops regulating gene and protein levels.

SFR6 May Act to Integrate Multiple Signals

sfr6 was originally isolated on the basis of its inability to cold-acclimate to tolerate freezing temperatures (Warren et al., 1996). *sfr6* expresses many cold-responsive (*COR*) genes to only very low levels (Knight et al., 1999) due to a failure in the interaction between the C-repeat binding factor (CBF; also known as the drought-responsive element binding factor 1; Stockinger et al., 1997; Liu et al., 1998) and the C-repeat (CRT/DRE) promoter element (Boyce et al., 2003). Already known as a gene essential for normal responses to low temperature, *SFR6* now appears to be part of a wider mechanism in the network regulating the circadian clock and flowering in response to external and internal changes such as light, metabolic status, and temperature. It has been known for many years now that flowering time is influenced by low temperature via processes including vernalization (Maclagan, 1933; Reeves and Coupland, 2000; Penfield, 2008).

More recently, it has become apparent that there is a relationship between flowering time and the pathways leading to cold acclimation (Kim et al., 2004; Yoo et al., 2007); therefore, the low temperature and circadian phenotypes of *sfr6* may interact at this level. Although the *sfr6* mutant expresses *CBF* genes to normal levels (Knight et al., 1999), the specific *COR* genes targeted by CBFs are not expressed normally, indicating a failure at some point in the CBF pathway. The *high expression of osmotically responsive genes1* mutant, which shows elevated transcript levels of the *CBF* genes and their targets, is early flowering (Lee et al., 2001), and, conversely, a putative nucleoporin mutant, Arabidopsis *nucleoporin160*, defective in CBF expression, is observed to flower late (Dong et al., 2006).

As already noted, the late flowering and low *CO* and *FT* aspects of the *sfr6* phenotype resemble plants with altered *GI* and *ZTL* expression (Fowler et al., 1999; Park et al., 1999; Somers et al., 2000; Kevei et al., 2006) and also *sex3*, a novel allele of *gi* that shows late flowering (Messerli et al., 2007). As *GI* is a ''hub'' in the regulation of clock and flowering time pathways, the changes we observe in its expression may well account for the delayed flowering, changes in clock-associated gene expression, and altered circadian phenotypes seen in *sfr6* plants. *GI* expression is known to be temperature sensitive (Gould et al., 2006; Paltiel et al., 2006); therefore, the changes in *GI* expression in *sfr6* may be linked to the mutant's altered response to temperature, and this remains to be investigated.

Our study shows that in addition to its role in cold gene expression and acclimation, SFR6 plays an important part in regulation of the circadian clock and in the control of flowering time. We have also demonstrated a role for sugars in clock regulation and we suggest that SFR6 may serve to integrate information such as temperature and sugar status to elicit a suitable response. Cloning and analysis of the *SFR6* gene may give insights into the mechanism by which this is achieved.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Chemicals

All experiments were carried out using the homozygous *sfr6* ethylmethanesulfonate mutant of the Arabidopsis (*Arabidopsis thaliana*) Col0 accession; Col0 plants were used as a wild-type control in all experiments. Except for flowering experiments, seedlings were grown on plates containing 1× MS salts with 0.8% to 1% agar and, where indicated, supplemented with 3% (w/v) Suc or isoosmolar concentrations of mannitol (96 mM) or 3-OMG (87 mM). In all cases, media pH was corrected to pH 5.8. Unless otherwise stated, seeds were surface-sterilized before being stratified in the dark at 4°C for 48 h prior to transfer to the growth chamber. Plants were grown in Sanyo MLR-350 growth chambers at a constant temperature (20°C). The light level during photoperiods or constant light was 50 μmol m^{-2} s^{-1}. All chemicals were purchased from Sigma except for the agar used in the germination and hypocotyl experiments purchased from Duchefa Biochemie (m1002.0500). To determine the effects of mannitol, 3-OMG, or 2.9× MS on growth, plants were grown for 14 d on 1× MS-agar plates with appropriate supplementation for 14 d in LD 12:12 at 20°C.

The microarray experiment was performed on plants grown at a constant temperature of 20°C in an LD 16:8 photoperiod and has been described elsewhere (Garnet Affymetrix array http://affy.arabidopsis.info/narrays/experimentpage.pl?experimentid=194). A total of 500 bp of promoter sequence was analyzed for each gene that showed at least 2-fold higher expression in wild type than in *sfr6*. Only genes giving a ''present'' call in both *sfr6* and wild type were included in this analysis. Promoter sequences were analyzed using the regulatory sequence analysis tools pattern matching function (van Helden, 2003).

Flowering Time

Flowering time experiments were conducted in growth chambers in LD 16:8 photoperiods and 20°C constant temperature. Two-week-old seedlings were transferred to sterilized soil following surface sterilization of seeds, stratification, and germination of seedlings on 1× MS plates without Suc. Plants were checked daily until the floral meristem was clearly visible. At this point, plants were dissected to count the total number of rosette leaves.

Real-Time Reverse Transcription-PCR

Col0 and *sfr6* seedlings were grown on MS-agar plates, as described above, with and without Suc, in LD 12:12 photoperiods for 12 d postgermination (clock gene expression experiments). At dawn on day 13, seedlings were transferred to LL. Samples of 20 to 30 seedlings were collected every 3 h and snap-frozen in liquid nitrogen; the first sample was collected at subjective dawn on day 14, after 24 h in LL. Similarly, for analysis of flowering time gene expression, seedlings were grown on plates as described above in LD 16:8 photoperiods for 13 d and sampled every 2 h on day 14 in a free-running cycle. RNA was extracted (RNeasy kit, Qiagen [74904] with additional DNAse digestion) from each sample and cDNA synthesized (Taq-Man, Applied

Biosystems [N808-0234] reverse transcriptase kit) for each time point. Real-time reverse transcription (RT)-PCR was carried out in triplicate with SYBR Green PCR Master mix (Applied Biosystems [4309155]) using an Applied Biosystems Prism-7300. Levels of specific mRNA and *βTUBULIN4* (*βTUB4*) controls were calculated by the standard curve method (Applied Biosystems user bulletin 2); the relative expression (arbitrary units) of each gene of interest was obtained by dividing by contemporaneous *βTUB4* expression. No-RT and no-template controls were included as negative controls for each set of reactions. Two or three independent biological repeats gave similar results.

Gene-specific primers were as follows: *CCA1*, forward 5′-TCTGTGTCTGACGAG GGTCGAATT-3′, reverse 5′-ACTTTGCGGCAATACCTCTCTGG-3′; *CO*, forward 5′-TGGCTCCTCAGGGACTCACTACAA-3′, reverse 5′-TTGACTCCGGCACAACACCAGT-3′; *FKF1*, forward 5′-TCTTGGTCGTAACTGTCGATTCC-3′, reverse 5′-GACGCCTTTGAGCTCGAGG-3′; *FT*, forward 5′-CCATTGGTTGGTGACTGATATCC-3′, reverse 5′-CTCATTGCCAAAGGTTGTTCC-3′; *GI*, forward 5′-CATTGCTGAGTTGGTCCGG-3′, reverse 5′-CCAGCACATCGTCTAAAAGTCG-3′; *TOC1*, forward 5′-ATCTTCGCAGAATCCCTGTGATA-3′, reverse 5′-GCACCTAGCTTCAAGCACTTTACA-3′; *βTUB4*, forward 5′-TTTCCGTACCCTCAAGCTCG-3′, reverse 5′-TGAGATGGTTAAGATCACCAAAGG-3′; and *ZTL*, forward 5′-CCGTCTTCGAAATGGTTACAGG-3′, reverse 5′-CTCTACATTGCAAGAAGCGGC-3′.

Leaf Movement

Seeds were surface-sterilized and stratified for 72 h at 4°C. Seedlings were grown on plates containing 1× MS salts with 2% agar with or without 3% Suc in LD 12:12 photoperiods at 22°C and were 11 d old at the commencement of imaging. Plants were assayed in constant light at 22°C, starting at subjective dawn 24 h after the discontinuation of the photoperiod; light level during photoperiods or constant light was 50 μmol m^{-2} s^{-1}. Circadian rhythms of leaf movement were measured by video imaging under constant light and analyzed using the BRASS interface (Brown, 2004; http://www.amillar.org/Downloads.html), as described previously (e.g. Edwards et al., 2005). Two (no Suc supplementation) or three (with Suc supplementation) independent biological repeats gave similar results, and the values are shown for all of these in Table I.

Analysis of Hypocotyl Length and Germination

Seeds were sown on square petri plates (120 cm) containing 1× MS with or without Suc and 1% agar, stratified as described above, and placed vertically in the growth chamber for 7 d in darkness or in constant light (50–60 μmol m^{-2} s^{-1}). All plates were subjected to a 2-h light treatment before the commencement of this period to allow seedlings in DD to germinate. After this period, plants were photographed and hypocotyl lengths were measured using ImageJ software (http://rsb.info.nih.gov/ij/). Germination rates were measured on horizontally grown MS agar plates as in all other experiments, with or without Suc, and in DD after an initial 2-h light period to allow germination. Germination was scored by checking for the emergence of a radical every 12 h.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *sfr6* takes longer to flower than wild type.

Supplemental Figure S2. Suc does not cause major osmotic stress to Arabidopsis seedlings.

Supplemental Figure S3. Expression of core clock components is reduced in *sfr6* seedlings growing in entrained conditions (LD 16:8).

Supplemental Table S1. List of 209 genes misregulated by at least 2-fold in *sfr6* when compared with Col0 wild type.

Supplemental Table S2. Timing of germination in DD of wild type and *sfr6*.

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