letters to nature

Electrophysiology

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The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*

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Many plants use day length as an environmental cue to ensure proper timing of the switch from vegetative to reproductive growth. Day-length sensing involves an interaction between the relative length of day and night, and endogenous rhythms that are controlled by the plant circadian clock[1]. Thus, plants with defects in circadian regulation cannot properly regulate the timing of the floral transition[2]. Here we describe the gene *EARLY FLOWERING 4* (*ELF4*), which is involved in photoperiod perception and circadian regulation. *ELF4* promotes clock accuracy and is required for sustained rhythms in the absence of daily light/dark cycles. *elf4* mutants show attenuated expression of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), a gene that is thought to function as a central oscillator component[3,4]. In addition, *elf4* plants transiently show output rhythms with highly variable period lengths before becoming arrhythmic. Mutations in *elf4* result in early flowering in non-inductive photoperiods, which is probably caused by elevated amounts of *CONSTANS* (*CO*), a gene that promotes floral induction[5].

In *Arabidopsis*, a facultative long-day plant, the floral transition occurs earlier when plants are grown in long days (LD) than when they are grown in short days (SD)[6]. *elf4* mutants flower early in SD and are therefore impaired in their ability to sense day length (Table 1). In addition, *elf4* mutants have elongated hypocotyls and petioles (Table 1), particularly in SD. Both of these phenotypes may result from defects in circadian regulation[7,8]. A hallmark of circadian regulation is the persistence of robust, accurate rhythms for many days under conditions of continuous light (LL) or continuous darkness (DD). Circadian outputs, including rhythmic leaf movements and the expression of chlorophyll *a/b*-binding protein (*CAB*) and cold- and circadian-regulated (*CCR*) genes, are markers of clock function.

We introduced the luciferase reporter gene fusions *CAB–LUC* and *CCR2–LUC* into wild-type and *elf4* mutant plants. *CAB* rhythms in wild-type persisted in LL, whereas *elf4* mutants lost rhythmicity after one 24-h cycle (Fig. 1a). The arrhythmia of the

Table 1 Flowering time and hypocotyl lengths of *elf4* mutants

	Total leaf number*		Hypocotyl length (mm)†		
	LD	SD	CL	LD	SD
Wild type	7.8 ± 0.4 (30)	30.6 ± 5.1 (28)	2.9 ± 0.7	2.2 ± 0.7	4.7 ± 1.3
elf4	6.4 ± 0.9 (36)	10.1 ± 1.1 (23)	2.4 ± 0.4	3.1 ± 0.7	9.0 ± 1.9
elf3-4	4.9 ± 0.3 (36)	5.9 ± 0.3 (23)	2.8 ± 0.6	6.0 ± 0.2	10.2 ± 2.2

*Mean ± s.d. total leaf number was counted as rosette leaves plus cauline leaves. The number of plants is given in parentheses.
† About 15 plants were analysed in each trial.
CL, continuous light; LD, 16 h light/8 h dark; SD, 8 h light/16 h dark.

population (Fig. 1a) concealed weak rhythms of variable period in individual *elf4* seedlings: many *elf4* seedlings had shorter or longer periods than those of wild-type seedlings (Fig. 1b). Unlike *CAB–LUC*, *CCR2–LUC* maintains a robust pattern of expression in DD. *elf4* seedlings showed weak *CCR2* expression rhythms of variable period in DD (Fig. 1d), which, like *CAB*, seemed arrhythmic in the population mean (Fig. 1c). Expression of *CCR2* also had variable periods in individual seedlings in LL (data not shown). For each marker, the rhythms of individual *elf4* seedlings damped before the rhythms of wild-type seedlings (data not shown).

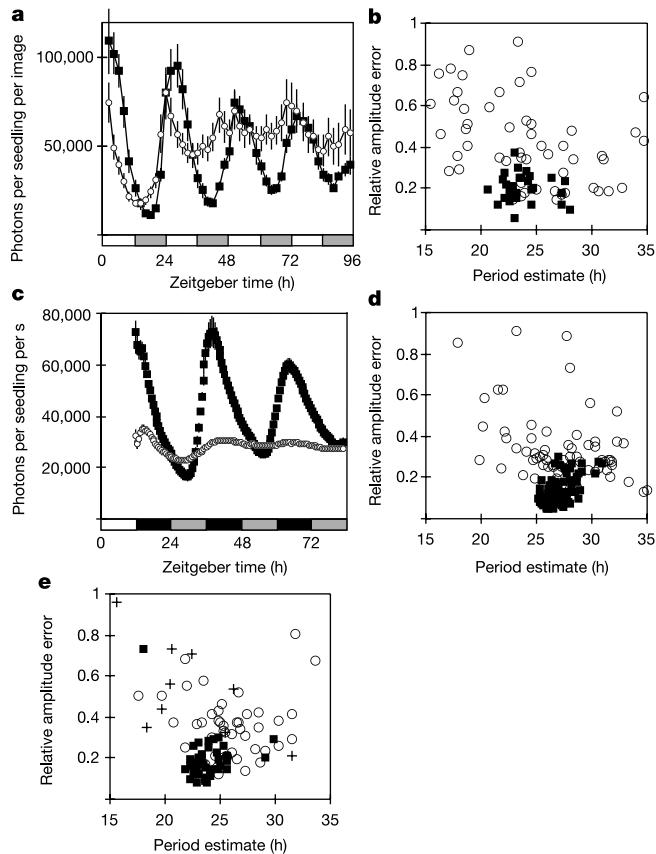Leaves of *Arabidopsis* show circadian movements that result in a

Figure 1 Circadian rhythms of *elf4* in LL or DD. **a**, *CAB–LUC* activity in plants transferred to LL at 0 h (predicted dawn). The transgene was crossed into *elf4*. Data are the means ± s.e.m. of eight individual seedling traces. **b**, *CAB–LUC* activity in LL in individual seedlings. The period estimate for each seedling is plotted against the relative amplitude error, a measure of rhythmic robustness that varies from 0 (a perfect cosine wave) to 1 (not statistically significant). Wild-type seedlings (30/30) were strongly rhythmic with a period of 23.8 ± 1.6 h (mean ± s.d.), whereas *elf4* seedlings (55/71) were weakly rhythmic with a period of 22.3 ± 5.1 h. **c**, *CCR2–LUC* activity in plants transferred to DD at 12 h (predicted dusk). At least 40 plants per line were tested for three independently transformed wild-type lines and four *elf4* lines. Data are normalized to the genotype mean; error bars show the standard deviation of each line from the genotype mean. **d**, Analysis of *CCR2–LUC* activity in DD. Wild-type (87/87) and *elf4* seedlings (67/89) were rhythmic, with periods of 26.4 ± 0.8 h and 27.1 ± 4.1 h, respectively. **e**, Rhythmic leaf movement in LL. Wild-type (45/45) and *elf4* (50/60) traces were rhythmic, with periods of 25.1 ± 1.3 h and 25.9 ± 3.2 h, respectively. In *elf3*, only 9 out of 20 traces were rhythmic, with a period of 22.5 ± 4.8 h. Filled squares, wild type; open circles, *elf4*; plus signs, *elf3*. **a** and **c** are representative of two independent experiments; **b**, **d**, **e** show pooled data representing three, two and three experiments, respectively. Grey bars represent subjective day and subjective night in **a** and **c**, respectively.

horizontal position during the day and a more vertical position at night. A large percentage of *elf4* seedlings showed rhythmic leaf movements for the first 1–2 d in LL; however, these rhythms were less robust than in wild type and were no longer detectable after 3 d (Supplementary Information Fig. 1). Similar to gene expression studies, the period of leaf movement in *elf4* varied greatly among individuals (Fig. 1e). *ELF4* is therefore important for the maintenance and accuracy of circadian rhythms under LL and DD, as tested through different outputs.

Early flowering, hyperelongation and impaired circadian rhythms in LL are all characteristics of *elf3* mutants[9,10]. In wild-type plants, *ELF3* sustains rhythmicity in long photoperiods and in LL by inhibiting phototransduction at dusk[11–13]. In contrast to *elf4*, *CAB–LUC* and *CCR2–LUC* expression in *elf3* mutants resembles that of the wild type in DD[13,14]. Also, *CAB–LUC* expression and leaf movement become arrhythmic immediately under LL in *elf3* mutants[8,15], rather than after one cycle as in *elf4* mutants (Fig. 1a). Thus, the function of *ELF4* is distinct from that of *ELF3*.

Although the *elf4* phenotype is unique in plants, the phenotype is similar to *frequency* (*frq*) mutants of *Neurospora crassa*. *Frq* participates in a rhythmic, transcriptional feedback loop that is essential for normal circadian rhythmicity[16]. Mutants that are null for *frq* can seem arrhythmic in constant conditions, but weak conidiation rhythms have been detected in these mutants, with periods that are distributed broadly about the wild-type mean[17]. This resembles the phenotype that we have shown for *elf4*. It is unclear whether *Frq* principally generates rhythms or organizes *Frq*-independent oscillators[16]. In an analogous fashion, *ELF4* could function in either process in the plant clock.

Arabidopsis genes that are thought to function in the circadian oscillator include *TIMING OF CAB EXPRESSION 1* (*TOC1*), which is expressed early in the night[18], and *CCA1*, which is expressed
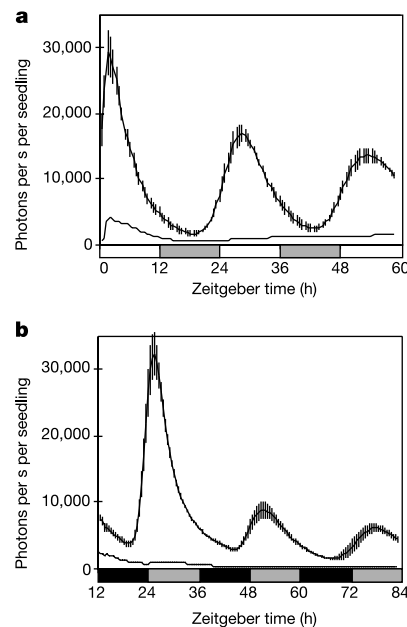

Figure 2 Regulation of *CCA1* expression by *ELF4*. Wild-type (line with error bars) and *elf4* (continuous line) plants containing a *CCA1–LUC* fusion assayed in LL (**a**) or DD (**b**). At least 18 plants per line in **a** or at least 40 plants per line in **b** were tested for five independently transformed wild-type and six *elf4* lines. *CCA1* expression was low in *elf4* (in LL: wild-type, 8,938 ± 1,631 counts per seedling per s (mean ± s.e.m.); *elf4*, 1,330 ± 478; in DD: wild-type 7,178 ± 927; *elf4*, 613 ± 156). Data are normalized to the genotype mean and are representative of two independent experiments.

shortly after dawn[4]. The *elf4* lesion disrupted rhythmic *CCA1* expression, showing the importance of *ELF4* in circadian regulation. The expression of a *CCA1–LUC* reporter gene was low and arrhythmic in *elf4* seedlings under both LL and DD (Fig. 2). *CCA1* expression in *elf4* occurred only through acute light activation after the dark/light transition (Fig. 2a). This low amplitude pulse of *CCA1* might have supported the single cycle of *CAB–LUC* and *CCR2–LUC* expression in *elf4* in LL (Fig. 1a and data not shown), before the normal rhythm was lost around subjective dawn of the subsequent cycle, when *CCA1* was again required but was not expressed. We propose that the circadian function of *ELF4* is to activate light-inducible clock components around dawn: *CCA1* could be one such component. *TOC1* may carry out a similar activation function[3].

elf4 was derived from a population mutagenized with the transfer DNA (tDNA) of *Agrobacterium tumefaciens*[19]. Adjacent to the tDNA was a deleted region predicted to contain a small open reading frame (ORF). A clone containing this ORF was sufficient to restore the phenotypes of *elf4* to that of wild type (Supplementary Information Fig. 2). In LL, the *ELF4* transcript maintains a robust rhythm, which shows that transcript abundance is under circadian control (Fig. 3a,

b). Robust cycling is not detectable after transfer to DD (Fig. 3a, c), which is similar to what has been observed for *TOC1* and *ELF3* (refs 12, 18). *ELF4* also shows a photoperiod-specific pattern of expression. In LD, messenger RNA levels peak around 12 h after dawn (Fig. 3d, e). In SD, the peak shifts to around 8 h after dawn (Fig. 3d, e). The predicted *ELF4* protein is 111 amino acids and does not contain known protein signatures (Supplementary Information Fig. 3). So far, organisms others than plants have not been found to contain genes similar to *ELF4*.

The circadian clock is thought to function in the floral transition through a coincidence mechanism in which inductive stimuli must be present during a particular phase of the rhythm to be effective[1]. Given its strong effect on circadian outputs, *ELF4* could repress flowering by controlling the phase of an output rhythm specific for flowering-time regulation. The floral-promoting gene *CO* may act in such an output[5,20,21]. The diurnal pattern of *CO* mRNA accumulation is distinct in inductive versus non-inductive photoperiods, and this expression pattern could contribute to a coincidence mechanism[21]. Several circadian rhythm mutants affect *CO* expression in ways that correlate with their early or late flowering phenotypes[21]. In SD-grown *elf4* mutants, the level of *CO* mRNA is increased at all time points (Fig. 3f, g). Increased *CO* expression is consistent with the early flowering *elf4* phenotype[21].

The molecular circuitry that comprises the *Arabidopsis* circadian clock is just beginning to be understood[3]. Our gene expression and leaf movement assays have revealed unique phenotypes of *elf4*, which indicate that *ELF4* functions either in a circadian oscillator or by conferring accuracy and persistence on a separate oscillator. Despite a unique role in clock regulation, the effect of *ELF4* on flowering time may occur by a mechanism similar to that of other circadian mutants, that is, through the misexpression of *CO*[21]. Further characterization of the *elf4* mutant will hopefully elucidate how components of the circadian machinery collectively control rhythmic processes throughout the day/night cycle, including the ability of plants to respond to photoperiod. □

Methods

Plant material and growth and conditions

All experiments were carried out on the Ws ecotype. Plants were grown in LD (16 h light/8 h dark) or SD (8 h light/16 h dark) under cool white light ($\sim 120 \mu mol\, m^{-2}\, s^{-1}$) at a constant 22 °C unless indicated otherwise. Hypocotyl-growth assays were done as described[22].

Analysis of *ELF4* and *CO* mRNA abundance

We isolated RNA from 1-week-old whole seedlings using TRI reagent (Sigma). Complementary DNA was synthesized by polymerase chain reaction with reverse transcription (RT–PCR) using 5 µg of total RNA, an oligo dT primer with an M13 sequence attached to the 5′ end and Superscript II reverse transcriptase (Gibco). As *ELF4* is a single exon gene, RT–PCR was carried out using a gene-specific primer, 5′-TATGAAGA GGAACGGCGAGA-3′, and the M13F primer, 5′-GTAAAACGACGGCCAGTCCC-3′, to prevent DNA contamination. *UBQ10* (ref. 23) was amplified as a control. We used 25 and 20 cycles for *ELF4* and *UBQ*, respectively. PCR fragments were transferred to a nylon membrane (Hybond) and hybridized with labelled *ELF4* and *UBQ* cDNAs. We measured *CO* mRNA abundance as described[21]. RNA blots contained 10 µg of total RNA per lane on a denaturing formaldehyde gel (1% agarose). RNA was transferred to a nylon membrane and probed with an *ELF4* antisense RNA probe.

Circadian rhythm assays

The *CAB2–LUC* construction has been described[24]. The DNA regions immediately upstream of the *CCR2* and *CCA1* genes were amplified by PCR from *Arabidopsis* (Ws-2) genomic DNA. These promoters were fused to luciferase and introduced into *Arabidopsis*. Seeds were stratified (48 h at 4 °C) on Murashige and Skoog (MS) agar medium with 3% sucrose. Seedlings were entrained for 6 d in 12 h light (140 µmol m⁻² s⁻¹ cool white fluorescent light)/12 h dark in controlled environment chambers (Percival or Sanyo) before being introduced into assay conditions[24]. The luminescence of individual seedlings was measured by low-light video imaging using a liquid nitrogen cooled camera (Roper Scientific)[24] or by counting in a Packard Topcount[25]. Instrument background has been subtracted from the data presented here. Periods were estimated starting from 24 h after the last dark/light transition, using FFT-NLLS software as described[11]. We entrained seedlings for leaf movement assays for 9 d as described above. Leaf growth was monitored by video imaging under LL. Movements of the first primary leaves over the first 90 h of imaging were analysed using FFT-NLLS software as described[8]. Period means and
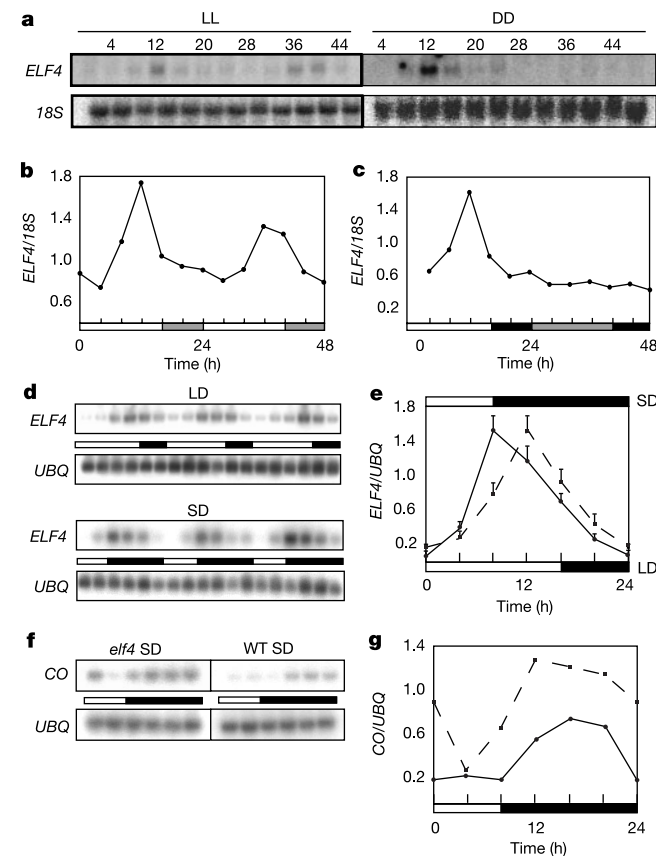

Figure 3 Expression of *ELF4* and *CONSTANS*. **a**, RNA blot of *ELF4* expression in LL and DD. Data are representative of two independent experiments. **b**, **c**, Quantification of *ELF4* expression in LL and DD. White and black rectangles indicate lights on and lights off, respectively. Grey rectangles indicate subjective day and subjective night in **b** and **c**, respectively. **d**, RT–PCR showing *ELF4* expression under LD and SD over 3 d. **e**, Trace showing *ELF4* expression in LD (dashed line) and SD (solid line). Points and error bars indicate the mean ± s.e.m. of three trials for LD and six trials for SD. **f**, RT–PCR showing *CO* expression in SD-grown wild-type (WT) and *elf4*. **g**, Trace showing *CO* expression in wild type (solid line) and *elf4* (dashed line). Data are representative of three independent experiments. The 0-h time point is plotted again at 24 h. White and black rectangles indicate lights on and lights off, respectively.

standard deviations are variance weighted. Rhythmic traces are those with any period estimate in the circadian range, 15–35 h, as described[8]. All rhythm assays were conducted at 22 °C.

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Protective role of phospholipid oxidation products in endotoxin-induced tissue damage

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Lipopolysaccharide (LPS), an outer-membrane component of Gram-negative bacteria, interacts with LPS-binding protein and CD14, which present LPS to toll-like receptor 4 (refs 1, 2), which activates inflammatory gene expression through nuclear factor κB (NFκB) and mitogen-activated protein-kinase signalling[3,4]. Antibacterial defence involves activation of neutrophils that generate reactive oxygen species capable of killing bacteria[5]; therefore host lipid peroxidation occurs, initiated by enzymes such as NADPH oxidase and myeloperoxidase[6]. Oxidized phospholipids are pro-inflammatory agonists promoting chronic inflammation in atherosclerosis[7]; however, recent data suggest that they can inhibit expression of inflammatory adhesion molecules[8]. Here we show that oxidized phospholipids inhibit LPS-induced but not tumour-necrosis factor-α-induced or interleukin-1β-induced NFκB-mediated upregulation of inflammatory genes, by blocking the interaction of LPS with LPS-binding protein and CD14. Moreover, in LPS-injected mice, oxidized phospholipids inhibited inflammation and protected mice from lethal endotoxin shock. Thus, in severe Gram-negative bacterial infection, endogenously formed oxidized phospholipids may function as a negative feedback to blunt innate immune responses. Furthermore, identified chemical structures capable of inhibiting the effects of endotoxins such as LPS could be used for the development of new drugs for treatment of sepsis.

Lipids containing polyunsaturated fatty acids such as 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) are especially prone to oxidative modification. Oxidation of PAPC results in generation of a mixture of oxidation products (OxPAPC), some of which stimulate adhesion of monocytes to endothelial cells and induce expression of inflammatory genes in endothelial cells[8–10]. Thus, a role for oxidized phospholipids as culprits in chronic inflammation was suggested. On the other hand, we have shown that OxPAPC inhibited neutrophil adhesion to endothelial cells induced by LPS[8].

Here we show that OxPAPC blocked LPS-induced upregulation of the inflammatory adhesion molecules E-selectin, ICAM-1 and VCAM-1 on human umbilical-vein endothelial cells (HUVEC) (Fig. 1a). The inhibitory effect of OxPAPC was concentration-dependent, with half-maximal inhibition observed at 10 μg ml^{-1} OxPAPC (Fig. 1b). The effects of LPS at concentrations as high as 500 ng ml^{-1} were inhibited by 50 μg ml^{-1} OxPAPC. Because expression of E-selectin, ICAM-1 and VCAM-1 is NFκB-dependent, we examined the influence of OxPAPC on signalling events at different levels of the NFκB cascade. We found that OxPAPC inhibited LPS-induced activation of a 5 × NFκB–luciferase reporter construct (Fig. 1c), binding of p65 to its DNA consensus site (Fig. 1d), and phosphorylation and degradation of IκBα (Fig. 1e).

NFκB is activated during inflammation by various receptors including the LPS toll-like receptor 4 (TLR4), the interleukin 1 (IL-1) receptor, and the tumour-necrosis factor-α (TNFα) receptor. In contrast to LPS, TNFα-induced phosphorylation and degra-

Supplementary figure 1

Leaf movements in *elf4*. Sample leaf traces showing that leaf movement rhythms in LL are **a** strong in wild type and **b** weak in *elf4* mutants.
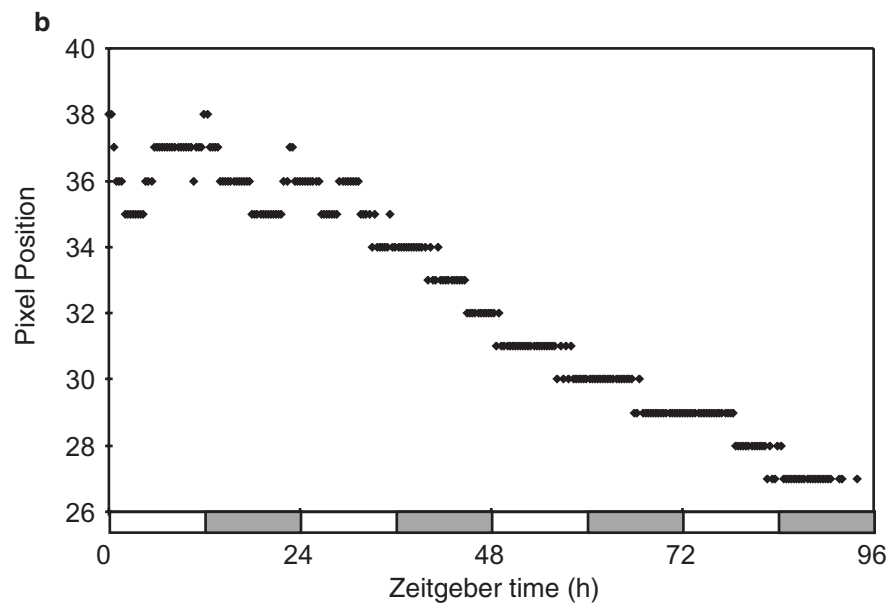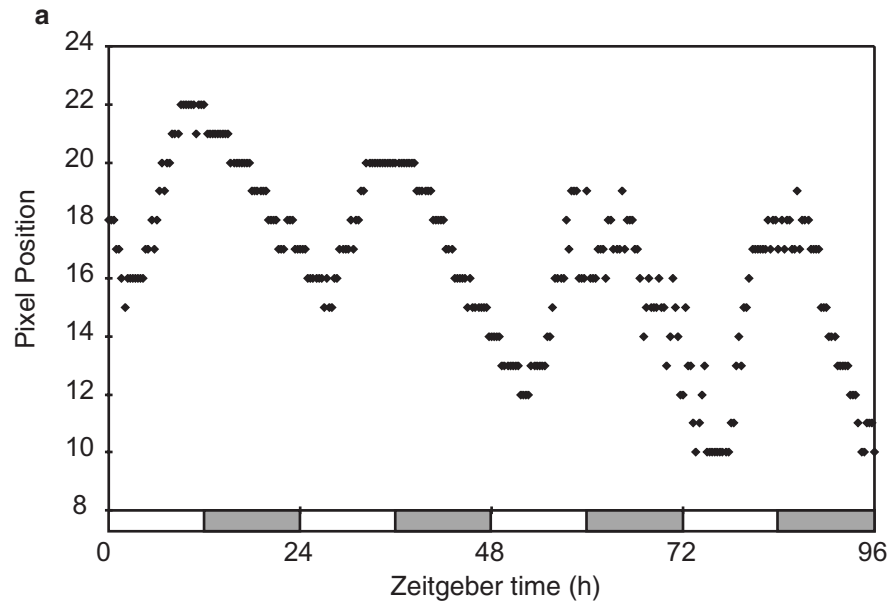

Supplementary figure 2

Restoration of *elf4* phenotypes.

a Fragment used to fully restore: **b** the photoperiod response,

c hypocotyl elongation in SD, **d** robust rhythmic leaf movement

(5 representative traces from the rescued plants are shown; 38/38 traces were rhythmic with mean relative amplitude error of 0.16, compared to WT, 0.19 and elf4, 0.37).

Supplementary Figure 3

Comparison of predicted amino-acid sequences of *ELF4* (At2g40080) and family members. This gene family currently includes *ELF4Os* from rice, *ELF4Sb* from Sorghum , and *ELF4*, At1g17460, At1g72630, and At2g29950 from Arabidopsis. Identical and similar residues are in reverse type or gray boxes, respectively. Numbers at the left indicate the respective amino-acid residues.

Amasino_supplementary figure 1 (A01248A)

a

b

Amasino_supplementary fig 2 (A01248A)

a

T28M21 (Chromosome II)

En/Spm-like transposon protein   ABC transporter

3'   5'   pMD24

BglII   584bp   *ELF4* 336bp   277bp   NcoI

b

2cm

elf4 + pMD24   *elf4*

c

Hypocotyl length (mm)

WT   *elf4*   *elf4*
                    +pMD24

d

Leaf position (arbitrary units)

Time in constant light (h)
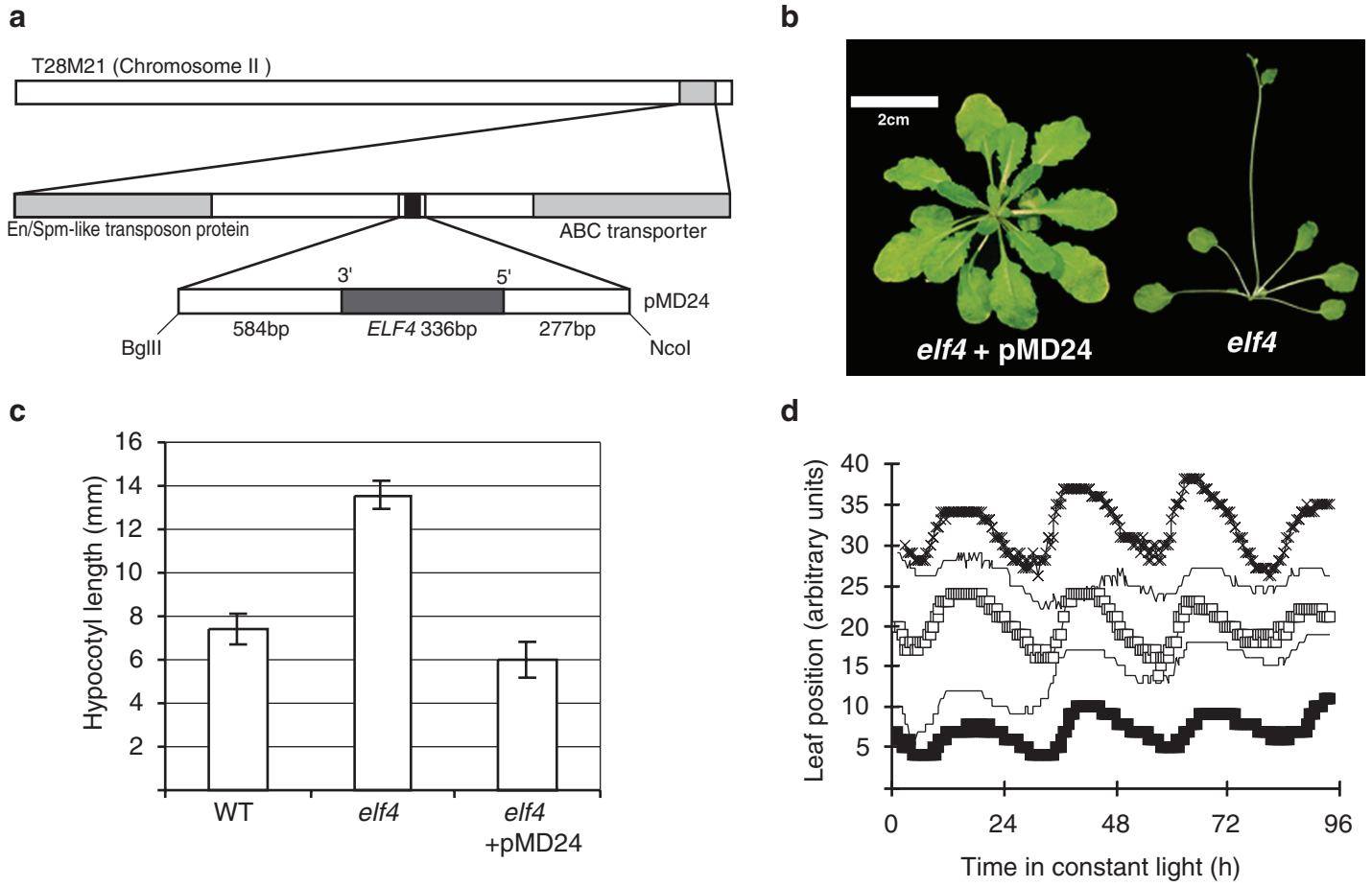
amasino_supplementary figure 3 (A01248A)

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ELF4Os      1  ------------------MEGDSFSGMANGGQVDNKLIQTFHKSFVQVQS
ELF4Sb      1  ------------------------MANGGGQAVDGKLIQTFHKSFVQVQS
At1g17460   1  ------------------MEGDVLSGFGDRHNMDGKLLQSFQKSFVDVQD
At1g72630   1  ------------------MEGDVYSGFGERYQMDGKLLQNFQKSFVQVQD
ELF4        1  -----------MKRNGETKRRRNVAEEAEQGEDPAMWENLDRNFRQVQS
At229950    1  MEASRNRSLVGNNRSPEMNENDGEDVAASAAVEDVEVWDTLSNGFKRAQL


ELF4Os     33  ILDQNRMLINEINQNHESRAPDNLTRNVGLIRELNNNIRRVVGLYADLSA
ELF4Sb     27  LLDQNRMLISEINQNHESRAPDNLTRNVGLIRELNNNIRRVVGLYADLSA
At1g17460  33  ILDQNRLLINEINQNHESKQPDNLGRNVGLIKELNNNIRRVASLYGDLSH
At1g72630  33  ILDQNRLLINEINQNHESKQADHLGRNVGLIRELNNNIRTVASLYGDLSH
ELF4       39  VLDRNRSLIQQVNDNHQSRMADNMSKNVALIQELNGNISKVVNMYSDLNT
At229950   51  YLDQNRDLIQRVNENHMSRIPDNVSRNVGLINEINGNISQVMEIYSDLSL


ELF4Os     83  SFARTMDASSEGDSSGTLR--SSDGAGRTGQKRVRPG--------------
ELF4Sb     77  SFARTMDASSDGDSSGTAIRSSAAAAAAGHKRIRWDGWKNFGWVTKQGY
At1g17460  83  SFARSVDASSEGESSGTLK----SDGKAN-QKRFRSDISLFADVATSRSF
At1g72630  83  SFARSVDASSEGESTGTLK----SDGKANNQKRFRSG-------------
ELF4       89  SFSSGFHGGKNGHDGGGAAG----------TRA----------------
At229950  101  NFAKKFDQRRRTTKDGDTT------TTTTGS-------------------


ELF4Os         --------------
ELF4Sb     127  SSKHLEWKLFAAQF
At1g17460  128  PHNYTDPNWHSEIE
At1g72630       --------------
ELF4            --------------
At229950        --------------
```