Original Article

A fast circadian clock at high temperatures is a conserved feature across Arabidopsis accessions and likely to be important for vegetative yield

Jelena Kusakina1,2, Peter D. Gould1 & Anthony Hall1

1Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK and 2School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK

ABSTRACT

The circadian clock is an endogenous 24 h oscillator regulating many critical biological processes in plants. One of the key characteristics of the circadian clock is that it is buffered against temperature, maintaining an approximately 24 h rhythm over a broad physiological temperature range. Here, we tested temperature-buffering capacity of the circadian clock across a number of Arabidopsis accessions using several circadian clock reporters: leaf movement, CCA1::LUC and LHY::LUC. We found that leaf movement was the best temperature buffered circadian output. On the other hand, when temperature increases, circadian rhythms of CCA1 and LHY transcription shorten considerably across all accessions, indicating that the clock driving expression of CCA1 and LHY is not perfectly buffered. This feature might be crucial to plants growing in a constantly changing environment, and here, we provide insight into the importance of period shortening to plant growth performance and the benefits of a flexible clock.

Key-words: Arabidopsis thaliana; natural variation; performance.

INTRODUCTION

The Earth’s rotation on its axis results in a 24 h light/dark cycle. The endogenous circadian clock is an adaptation to this cycle, allowing an organism to time events within this 24 h period (Dunlap 1999; Harmer 2009). In eukaryotes and some prokaryotes, a large proportion of critical biological processes are under circadian control, including nitrogen fixation in bacteria, conidiation in fungi, photoperiodic regulation of flowering time in plants and sleep/wake cycles in humans (Pittendrigh et al. 1959; Mills, Minors & Waterhouse 1974; Kondo et al. 1993; McClung 2001; Albrecht 2002). It is an adaptive advantage to have an endogenous rhythm that matches the periodic environment so that organisms are able to correctly anticipate the time of the day (Ouyang et al. 1998; Green et al. 2002; Dodd et al. 2005). For example, deviation of the photoperiod from the 24 h cycle negatively affects growth of tomato plants, and prolonged exposure of these plants to continuous light significantly damages them (Highkin & Hanson 1954). Furthermore, due to the correct anticipation of dawn, Arabidopsis thaliana short- and long-period mutants performed better when their circadian clocks matched the external light/dark environment, fixing more carbon and accumulating more biomass (Dodd et al. 2005).

The eukaryotic circadian clock consists of interacting genes and proteins forming interlocking negative/positive feedback loops (Dunlap 1999). According to the current model, the central plant oscillator of the Arabidopsis circadian clock is made up of two coupled transcriptional feedback loops, that is, morning and evening (Pokhilko et al. 2012). The morning loop involves PSEUDO RESPONSE REGULATOR 7 and 9 (PRR7, PRR9) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Alabadi et al. 2001; Gardner et al. 2006). CCA1 and LHY are MYB transcription factors that regulate PRR7 and PRR9, which in turn inhibit CCA1 and LHY. CCA1 and LHY also bind directly to the TIMING OF CAB EXPRESSION 1 (TOC1) promoter from the evening loop and inhibit its transcription, thus coupling the morning and evening loops (Gendron et al. 2012; Pokhilko et al. 2012).

Temperature massively influences plant growth and survival, and the consequences are especially crucial for commercially grown plants with considerable effects on yield (Law & Crafts-Brandner 1999; Tonsor et al. 2008; Zinn, Tunc-Ozdemir & Harper 2010). It is well-known that circadian rhythms are temperature compensated, that is, the clock is well buffered against temperature changes and continues to oscillate with approximately the same period (Pittendrigh 1954). The temperature compensation mechanism is not yet completely understood, but the latest work supports the involvement of CCA1 and LHY in combination with other genes, for example, G1 (GIGANTEA), PRR7 and PRR9 (Gould et al. 2006; Salome, Weigel & McClung 2010). Due to the high genetic similarity of CCA1 and LHY, it was thought that these clock components have redundant functions in circadian regulation. However, using a CAB2::LUC reporter gene in cca1 null and lhy null mutants, it was demonstrated that at different temperatures, the roles of CCA1 and LHY differentiate (Gould et al. 2006). Functioning CCA1 is of more significance in buffering the clock at low temperatures while LHY is more important at high temperatures (Gould et al. 2006).
Leaf movement assays have previously revealed that there is considerable variation in circadian period and its temperature-buffering capability between Arabidopsis accessions (Edwards et al. 2005; Michael et al. 2003a; Swarup et al. 1999; reviewed in Anwer & Davis 2013). Here, we investigated natural variation in circadian clock performance in response to increased temperature by examining two circadian clock genes that have recently been suggested to be members of the clock temperature-buffering mechanism, CCA1 and LHY. We found that the circadian period of CCA1 and LHY transcription shortened at increased temperature. While CCA1 period shortening was uniform across all accessions, LHY period change was accession specific. In addition, preliminary growth performance experiments suggested that a temperature-responsive clock is more beneficial for plants than a temperature-buffered one.

MATERIALS AND METHODS

Arabidopsis accessions

Seed for all accessions were ordered from the Nottingham Arabidopsis Stock Centre (NASC) or from the Arabidopsis Biological Resource Center (ABRC): An-1 (N6603), C24 (N28126), Col-0 (N1093), Cvi (N8580), Eri (CS22548), Kyo (N3964), Ler (N24596), Je54 (N924), Or-0 (N1432), Wc-1 (N1588), Dog-5 (N22699), Phw-1 (N6019), Ct-1 (N6674), Est (N1148), Fei-0 (CS22645), Van-0 (N1585), Ws-2 (N1601). Information on geographical location for all 18 Arabidopsis accessions from this study is presented in Table 1.

The CAB2::LUC, TOC1::LUC, CCA1::LUC and LHY::LUC transgenes in pPCV812 were obtained from Lázló Kozma Bognár (McWatters et al. 2007). Agrobacterium tumefaciens strain GV3101 was used to transform all Arabidopsis accessions via the floral dip method (Clough & Bent 1998; Davis et al. 2009). Transformed plants were left to recover and senesce. Harvested T1 seed was screened on 1.5% agar 1x Murashige and Skoog (MS) plates containing 50 μg mL⁻¹ hygromycin. Selected resistant seedlings were allowed to self. Homozygous lines strongly expressing LUC activity were used in experiments with four independent transgenic lines for each construct for each accession.

Growth conditions

For the leaf movement assays, seeds were surface-sterilized in 70% ethanol for 1 min, 50% bleach with 0.01% Tween 20 for 10 min followed by one rinse in sterile water. For the bioluminescence assays, seeds were gas sterilized for 3 h in a glass desiccation jar holding 500 mL of reverse osmosis water with two dissolved chlorine tablets (CLO-TABS, Arrow Solutions, Swadlincote, UK) and with subsequent addition of 5 mL of hydrochloric acid (modified from Desfeux, Clough & Bent 2000). Seeds were then moved to a sterile flow hood for 1 h to allow any remaining chlorine to evaporate. Sterilized seeds were re-suspended in 0.15% agar and sown onto 1.5% agar MS plates either as individual seeds (for leaf movement) or in rows of eight small clusters of approximately 20–30 seeds (for luminescence). Seeds were stratified in the dark at 4 °C for 3 d before being moved to the 22 °C room and grown under 12:12 light:dark (L/D) cycles of 80–100 μmol m⁻² s⁻¹. Ten-day-old seedlings were used for all experiments.

Circadian rhythm analysis

For leaf movement, seedlings were imaged in Sanyo MLR350 plant growth chambers (Sanyo, Osaka, Japan) under constant

| Name   | Geographical location | Country     | Latitude (°) | Longitude (°) | Altitude (m) |
|--------|-----------------------|-------------|--------------|---------------|--------------|
| An-1   | Belgium               | Belgium     | N 51–52      | E 4–5         | 1–100        |
| C24    | Portugal              | Portugal    | n/a          | n/a           | n/a          |
| Col-0  | Germany               | Germany     | N 50         | E8            | 1–100        |
| Ct-1   | Italy                 | Italy       | N 37–38      | E 15          | 1–100        |
| Cvi    | Cape Verde Island     | Cape Verde  | N 15–17      | W 23–25       | 1200         |
| Dog-5  | Turkey                | Turkey      | N 38.3       | E 42          | 1503         |
| Eri    | Sweden                | Sweden      | N 56.4       | E 15.4        | n/a          |
| Est    | Estonia               | Estonia     | N 59         | E 26          | 100–200      |
| Fei-0  | Portugal              | Portugal    | N 40         | W 8           | 100–300      |
| Je-54  | Former Czechoslovakia | Former Czechoslovakia | N 50 | E 15 | n/a |
| Kyo    | Japan                 | Japan       | N 35.3       | E 135.9       | n/a          |
| Ler    | Germany               | Germany     | N 53         | E 15–16       | 1–100        |
| Or-0   | Germany               | Germany     | N 50.5       | E 7.6         | n/a          |
| Phw-19 | UK                    | UK          | N 51.2       | E 0.9         | n/a          |
| Sha    | Tajikistan            | Tajikistan  | N 39         | E 70          | 3400         |
| Van-0  | Canada                | Canada      | N 49–50      | W 123         | 1–100        |
| We-1   | Germany               | Germany     | N 53         | E 10          | 1–100        |
| Ws-2   | Belarus               | Belarus     | N 52         | E 30          | 100–200      |

Table 1. Geographical information for the 18 accessions used in the study

Information of collection sites was obtained from TAIR (http://www.arabidopsis.org).

n/a, information not available.
white light (25 μmol m⁻² s⁻¹) at either 17 or 27 °C (depending on the experiment) over the course of 1 week as described by Edwards et al. 2005. Images were taken by Sony Exwave HAD cameras (Soverein International, Southport, UK). Luminescence was imaged in a Sanyo MIR-553 incubator (Sanyo Gallenkamp, UK) with an ORCA-II-BT 1024 16-bit camera (Hamamatsu Photonics, Shizouka, Japan) cooled to -80 °C as described in Gould et al. 2006. For free-running experiments, LUC activity was monitored at 17 or 27 °C and continuous red/blue light (RBL; 20 μmol m⁻² s⁻¹ of blue light: 20 μmol m⁻² s⁻¹ of red light), provided by red/blue light-emitting diode (LED) arrays (MD Electronics, Coventry, UK). For diurnal experiments, LUC activity of CCA1, LHY, TOC1 or CAB2 reporter genes were monitored at 12, 17 or 27 °C in 12:12 L/D cycles of RBL. Images from leaf movement and luminescence were processed using Metamorph 6.0 image-analysis software (Molecular Devices, Wokingham, UK). Data from the first 24 h after the transfer to continuous light (LL) was not included in analysis. Period estimates and relative amplitude errors (RAE) were calculated in BRASS (available from http://www.amillar.org/downloads.html) by running a fast Fourier transformed non-linear least-square analysis program (Plautz et al. 1999). RAE is a measure of rhythm robustness that ranges from 0 (a perfect fit to the wave) to 1 (no fit). Period estimates were used to calculate 1/Q₁₀ (Q₁₀ is a temperature coefficient of reaction rate change in response to a 10 °C temperature shift) from the equation 1/Q₁₀ = (1/τ₁₀)−1/(1/τ₀), where τ and τ₁₀ were period estimates at 17 and 27 °C, respectively. In this study, Q₁₀ was an inverse of a real Q₁₀ for better visual representation of an increased oscillator speed, which is associated with a period shortening (Edwards et al. 2005).

Plant growth performance assay

Plant performance at 17 and 27 °C was evaluated by determining the dry weight of Arabidopsis seedlings. Seeds were sown onto soil, stratified at 4 °C and grown at 22 °C under 12:12 L/D conditions. After 14 d, seedlings were transplanted into 20-cell half trays with one seedling per cell. After 2 d recovery, transplanted seedlings were transferred to 17 °C or 27 °C Sanyo MLR350 growth chambers (Sanyo), with one tray representing each accession at each temperature. After 14 d, prior to bolting, all seedlings were harvested, dried in the 80 °C oven for 48 h and weighed. Weight gain was used to represent the weight increase associated with the 27 °C treatment and was calculated by subtracting the mean weight at 17 °C from the mean weight at 27 °C. Relative weight gain was calculated by dividing the weight gain between 17 and 27 °C by the weight at 17 °C.

Statistical analysis

Effects of accessions, markers and temperature treatments as well as accession*marker, accession*temperature, marker*temperature and accession*marker*temperature interactions were evaluated using analysis of variance (ANOVA) in MINITAB (Minitab Ltd, Coventry, UK) 15.1.30.0 software. Paired t-tests were used to assess the differences between 17 and 27 °C temperature treatments. All calculations were considered to be statistically significant at P < 0.05.

Sequence analysis

Sequences for the nine clock genes PRR9, CCA1, ELF4 (EARLY FLOWERING4), GI, LHY, LUX (LUX ARRHYTHMO), TOC1, ELF3 (EARLY FLOWERING3) and PRR7 were downloaded for 14 of the phenotyped accessions that had sequenced genomes and had been uploaded to the (http://signal.salk.edu/atg1001/index.php). Sequence analysis was performed using Geneious (http://www.geneious.com). Sequences were aligned using MUSCLE with default settings and for each gene, a phylogenetic tree was drawn using the Jukes Cantor model and the neighbour joining tree-building method with 1000 replicates.

RESULTS

Natural variation in temperature on circadian clock genes

CCA1 and LHY are key components of the Arabidopsis circadian clock, which have been proposed to have different roles in the temperature compensation mechanism despite their apparent redundancy under standard laboratory conditions (Gould et al. 2006). Monitoring expression of these genes should reveal if any variation in temperature buffering of the molecular clock is naturally present in Arabidopsis accessions and demonstrate the immediate response of the circadian oscillator to temperature change. CCA1 and LHY transcription was monitored using LUC reporter fusions in a variety of Arabidopsis accessions (described previously in Millar et al. 1995; Table 1). For each accession, four independent transgenic lines of the T₂ (second generation) were analysed for both CCA1::LUC and LHY::LUC reporter constructs (normalized average traces, Supporting Information Fig. S1). Prior to the experiment, seedlings were grown at 12:12 L/D and at dawn, they were transferred to continuous light (LL) at either 17 or 27 °C. At 17 °C the circadian period of CCA1 in tested accessions ranged from 23.9 to 25.2 h with μ = 24.7 h (μ = period average; Table 2). However, upon transfer of the plants to 27 °C, CCA1 period became significantly shorter (μ = 20.9 h, t-test, P < 0.001; Fig. 1). No accessions were able to keep the same period of rhythmicity at 27 °C as they did at 17 °C (P < 0.001), instead exhibiting 3.1–4.2 h period shortening with the temperature increase, dependent upon accession (Supporting Information Fig. S1). In addition to the period alteration, a slight overall decline in rhythm robustness was observed, with an average RAE value increasing from 0.2 at 17 °C to 0.3 at 27 °C (P < 0.001) (Table 2). RAE evaluates rhythm robustness and varies from 0 (robust rhythm) to 1 (no rhythm). Depending on accession, high temperature also induced an increase in the variance of period from 0.64 at 17 °C to 0.86 at 27 °C (F-test, P = 0.017; Fig. 2). The increase in variance indicates that the precision of
the circadian clock is negatively affected by the temperature increase. However, as seen in the RAE plots (Fig. 2), the variance increase was subtle, suggesting that at 27 °C the circadian clock oscillates robustly but with a shorter period.

At 17 °C, the \textit{LHY::LUC} circadian period of most accessions was close to 24 h (\(\mu = 24.5\) h) ranging from 23.8 h to 25.2 h (Table 2). Similar to \textit{CCA1::LUC}, the temperature increase caused considerable period shortening across all accessions (\(\mu = 27^\circ\text{C} = 21.3\) h; Supporting Information Fig. S1). However, the magnitude of the change was accession specific and ranged from \(-1.9\) h (e.g. C24) to \(-4.1\) h (e.g. Col-0; Table 2). In contrast to \textit{CCA1}, where seven accessions had more than a 4 h period decrease as the temperature changed from 17 to 27 °C, only three accessions (Col-0, Kyo and Ler) had a \(-4\) h period change for \textit{LHY}. This could suggest that the \textit{LHY} rhythm is often better temperature buffered than \textit{CCA1}. On the other hand, temperature had more impact on variance of \textit{LHY} period (period variance increased from 0.75 at 17 °C to 1.03 at 27 °C; \(F\text{-test, } P = 0.008\) than \textit{CCA1} (period variance increased from 0.64 at 17 °C to 0.86 at 27 °C; \(F\text{-test, } P = 0.017\); Table 2; Fig. 2). However, as in \textit{CCA1}, the effect was statistically significant but not very strong (Fig. 2). The 27 °C treatment influenced the robustness of \textit{LHY} rhythms (Fig. 1), where the average \textit{LHY} RAE increased from 0.16 at 17 °C to 0.38 at 27 °C (\(t\text{-test; } P < 0.001\); the average RAE for \textit{CCA1} was 0.15 at 17 °C and 0.25 at 27 °C). Interestingly, in \(\geq 80\%\) of cases, \textit{CCA1} oscillated faster than \textit{LHY} resulting in a shorter period. Overall, as with \textit{CCA1}, high temperature affected robustness and period of \textit{LHY}; however, the effect on \textit{LHY} was more accession specific. This is consistent with previous reports of functional differentiation of CCA1 and LHY as well as uncoupling of circadian feedback loops when subjected to high or low temperatures (Gould et al. 2006).

Analysis of variance was used to determine whether the change in period and rhythm robustness was affected significantly by the circadian marker choice and whether it was accession dependent. It was revealed that RAE was affected by all factors, that is, temperature, accession, choice of marker and their combinations (Table 3). In terms of the circadian

### Table 2. \textit{CCA1::LUC} and \textit{LHY::LUC} bioluminescence period estimates (±) and RAE for 17 and 27 °C

| Accession | Period (h) | SE | RAE | Period (h) | SE | RAE | Period (h) | SE | RAE | Period (h) | SE |
|-----------|-----------|----|-----|-----------|----|-----|-----------|----|-----|-----------|----|
| An1       | 25.0      | 0.1 | 0.2 | 20.8      | 0.3 | 0.2 | 4.2       |     |     |           |    |
| C24       | 25.1      | 0.1 | 0.2 | 21.6      | 0.4 | 0.3 | 3.6       |     |     |           |    |
| Col-0     | 24.8      | 0.1 | 0.2 | 20.7      | 0.1 | 0.2 | 4.1       |     |     |           |    |
| Cr-1      | 24.9      | 0.1 | 0.1 | 21.7      | 0.2 | 0.3 | 3.1       |     |     |           |    |
| Cvi       | 24.5      | 0.1 | 0.2 | 20.6      | 0.1 | 0.3 | 3.9       |     |     |           |    |
| Dog-5     | 25.1      | 0.0 | 0.2 | 21.7      | 0.1 | 0.2 | 3.4       |     |     |           |    |
| Eri       | 24.0      | 0.0 | 0.1 | 19.9      | 0.1 | 0.3 | 4.1       |     |     |           |    |
| Est       | 24.9      | 0.1 | 0.1 | 20.7      | 0.1 | 0.3 | 4.2       |     |     |           |    |
| Fei-0     | 25.0      | 0.2 | 0.2 | 21.6      | 0.1 | 0.3 | 3.4       |     |     |           |    |
| Je54      | 24.9      | 0.0 | 0.1 | 21.3      | 0.1 | 0.2 | 3.6       |     |     |           |    |
| Kyo       | 24.6      | 0.1 | 0.2 | 20.5      | 0.2 | 0.3 | 4.1       |     |     |           |    |
| Ler       | 24.3      | 0.0 | 0.1 | 20.0      | 0.1 | 0.2 | 4.3       |     |     |           |    |
| Or-0      | 24.2      | 0.3 | 0.2 | 20.6      | 0.3 | 0.3 | 3.6       |     |     |           |    |
| Phw-19    | 24.7      | 0.1 | 0.1 | 20.8      | 0.1 | 0.3 | 3.9       |     |     |           |    |
| Sha       | 24.0      | 0.1 | 0.2 | 19.9      | 0.2 | 0.3 | 4.1       |     |     |           |    |
| Van-0     | 25.0      | 0.2 | 0.1 | 21.5      | 0.2 | 0.3 | 3.6       |     |     |           |    |
| We-1      | 25.2      | 0.1 | 0.2 | 21.8      | 0.1 | 0.2 | 3.4       |     |     |           |    |
| Ws-2      | 24.8      | 0.0 | 0.2 | 21.6      | 0.1 | 0.2 | 3.2       |     |     |           |    |

SE, standard error; \(\Delta\), circadian period difference between 17 and 27 °C.

### Figure 1. Overview of \textit{CCA1} and \textit{LHY} temperature compensation for 18 Arabidopsis accessions. Period estimates for groups of seedlings are plotted against their relative amplitude error (Rel. Amp. Error) with squares representing \textit{CCA1::LUC} activity; triangles, \textit{LHY::LUC} activity; solid symbols, 17 °C data; empty symbols, 27 °C data. Groups of seedlings were grown under 12:12 L/D conditions at 22 °C and after 10 d transferred to continuous light and either 17 or 27 °C where \textit{CCA1::LUC} and \textit{LHY::LUC} luminescence rhythms were assessed. For each accession at each temperature, \(n = 16\).
Figure 2. Effect of temperature on CCA1 (squares) and LHY (triangles) in Arabidopsis accessions. Plants were grown on MS agar under 12:12 L/D for 10 d before the transfer to 17 °C (solid symbols) or 27 °C (empty symbols) and continuous light, at which CCA1::LUC and LHY::LUC rhythms were assessed. Scatter plots illustrate period estimates for each individual group of seedlings plotted against its Rel. Amp. Error. n = 16 for all accessions except Ws, where n = 4.

© 2013 The Authors. Plant, Cell & Environment published by John Wiley & Sons Ltd, 37, 327–340
period, both markers responded to the increased temperature similarly by shortening the period. However, the effect of the marker*temperature interaction was significant, indicating that there was a marker difference for the temperature treatments used. Indeed, while overall there was no large difference between the molecular markers at 17 °C (t-test; \( P = 0.05 \)), the difference became profound at 27 °C (t-test; \( P < 0.001 \)), which is well demonstrated in Figs 1 and 2. The accession*marker effect was also significant. For example, in accessions Eri, Ler, Van-0 and Wc-1, \( CCA1 \) and \( LHY \) oscillated with similar periodicity at 17 °C (t-test; \( P = 0.692, P = 0.317 \); 17° and \( P = 0.528 \); 17°; respectively); however, while temperature treatment affected \( CCA1 \) and \( LHY \) to the same degree in Ler (t-test; \( P = 0.084 \)) and Wc-1 (t-test; \( P = 0.344 \)), it caused period differentiation between the markers in Eri (t-test; \( P = 0.007 \)) and Van-0 (t-test; \( P = 0.001 \)).

To summarize, even though \( CCA1 \) and \( LHY \) have a short period at 27 °C in all accessions, the effect that the temperature increase has on each gene is accession dependent.

Natural variation in temperature buffering of leaf movement rhythms

We wished to investigate if variation in temperature buffering of the circadian clock observed at the molecular level underlies variation of the overt rhythms previously reported for different \textit{Arabidopsis} accessions \cite{Michael et al. 2003a, Edwards et al. 2005}. We examined temperature buffering of \textit{Arabidopsis} leaf movement rhythms, as it is one of the most common overt rhythms used by circadian researchers. For this, seedlings were grown under 12:12 L/D at 17 °C and after 10 d transferred to LL and appropriate temperature (17 °C or 27 °C), where their free-running circadian periods were measured. On average, at 17 °C all accessions had robust circadian leaf movement rhythms (mean RAE = 0.10) and a range of periods from 24.5 to 27.5 h (Fig. 3; Table 4). As temperature increased to 27 °C, there was a decrease in the circadian rhythmicity robustness (mean RAE = 0.16; Fig. 3). Furthermore, 27 °C caused an increase in the variance of

![Figure 3. Overview of leaf movement temperature compensation for 18 Arabidopsis accessions. Period estimates for individual leaves are plotted against their Rel. Amp. Error with black squares representing 17 °C and white squares 27 °C data. All seedlings were grown on MS agar under 12:12 L/D conditions at 22 °C and after 10 d transferred to continuous light and either 17 or 27 °C where their circadian leaf movement rhythm was assessed. For each accession at each temperature \( n = 30-40 \). (a) Leaf movement data for 17 °C; (b) leaf movement data for 27 °C and (c) leaf movement data combined for 17 and 27 °C.](image-url)
period (from 2.17 at 17 °C to 6.77 at 27 °C, F-test, P < 0.001) and RAE (from 0.003 at 17 °C to 0.010 at 27 °C, F-test, P < 0.001), suggesting that the precision of the circadian clock has been affected. In addition to the decrease in the clock precision, temperature increase also caused a general shift towards period shortening (μ = 25.7 h at 17 and 23.9 h at 27 °C; t-test, P < 0.001). This result is consistent with Edwards et al. (2005), who also reported a significant period decrease when assaying 27 Arabidopsis accessions at 27 °C in comparison to 22 °C.

Despite the general period shortening at 27 °C, we found that there was accession-specific variation in this response (Fig. 4; Table 4). Several accessions’ circadian period remained unchanged with the temperature increase (C24, Cvi, Je54 and Kyo; Table 4). However, unchanged period estimates did not always signify a completely temperature-buffered clock, which is clearly demonstrated by individual RAE plots (Fig. 4). For example, even though the average period for Kyo was statistically the same between 17 and 27 °C, the data on RAE plots appears more scattered. This is illustrated by the spread of data points along the X-axis due to the increase in period variance at 27 °C (from 0.52 at 17 °C to 9.16 at 27 °C; F-test, P < 0.001) in comparison to the tightly clustered points at 17 °C (Fig. 4). Temperature increase caused a significant period shortening in approximately 80% of all accessions tested (14 out of 18) (Table 4). Accessions An1, Cvi, Est, Fei-0 and Or-0 were the most sensitive to temperature, in terms of period shortening, displaying a more than 3 h period decrease when assessed at 27 °C versus 17 °C. Accessions Ct-1, Je54 and Kyo; Table 4). However, unchanged period estimates did not always signify a completely temperature-buffered clock, which is clearly demonstrated by individual RAE plots (Fig. 4). For example, even though the average period for Kyo was statistically the same between 17 and 27 °C, the data on RAE plots appears more scattered. This is illustrated by the spread of data points along the X-axis due to the increase in period variance at 27 °C (from 0.52 at 17 °C to 9.16 at 27 °C; F-test, P < 0.001) in comparison to the tightly clustered points at 17 °C (Fig. 4). Temperature increase caused a significant period shortening in approximately 80% of all accessions tested (14 out of 18) (Table 4). Accessions An1, Cvi, Est, Fei-0 and Or-0 were the most sensitive to temperature, in terms of period shortening, displaying a more than 3 h period decrease when assessed at 27 °C versus 17 °C (Table 4). However, despite the period decrease, temperature did not affect the clock’s precision in An1 (period variance of 3.16 at 17 °C, 7.19 at 27 °C, F-test, P = 0.09), Est (period variance of 2.98 at 17 °C, 4.11 at 27 °C, F-test, P = 0.482) and Or-0 (period variance of 2.58 at 17 °C, 1.56 at 27 °C, F-test, P = 0.312). Conversely, in addition to the period shortening, Cvi and Fei-0 exhibited reduced precision of the clock with period variance changing from 1.71 at 17 °C to 12.09 at 27 °C (F-test, P < 0.001) for Cvi and from 0.26 at 17 °C to 10.94 at 27 °C for Fei-0 (F-test, P < 0.001; Fig. 4). In 9 out of 18 accessions, circadian periods changed by 1–2 h and, as previously, the effect on period robustness and clock precision was accession specific (Fig. 4). Overall, natural variation in response to elevated temperature was observed in period and robustness of Arabidopsis circadian leaf movement, suggesting accession-specific differences in the circadian clock.

### Increased temperature causes changes in the diurnal expression pattern of key clock genes

Collectively, this study of both rhythms in circadian clock genes and overt circadian rhythms (i.e. leaf movement) has identified a negative relationship between temperature and period length, where period shortens with increasing temperature. A negative relationship between temperature and circadian period has also been observed in cotyledon movement of Brassica rapa (Lou et al. 2011), suggesting that increases in temperature cause period shortening in plants other than Arabidopsis. Period shortening may be an adaptation to warmer temperatures allowing certain clock-controlled aspects of physiology or biochemistry to finish earlier in the photoperiod thus avoiding the hotter parts of the day.

While analysis of free-running rhythms allows us to identify small changes in period in the absence of other factors perturbing the rhythms, another important question is how do changes in free-running period alter the diurnal expression of clock-regulated genes? To investigate this, plants of the Col-0 background expressing LHY, CCA1, TOC1 or CAB2::LUC were grown under 12:12 L/D at 22 °C. They were then transferred to 12, 17 or 27 °C, with the 12:12 L/D...
continued. Both of the morning genes, \( LHY \) and \( CCA1 \), displayed a rapid and sharper increase in their promoter activity following dawn at 27 °C compared to 12 and 17 °C (Fig. 5 and Supporting Information Fig. S2). In addition, the trough in their expression was lower. For \( LHY::LUC \), there is also a much lower level in expression at 27 °C with its peak being of a similar level to trough expression at 17 °C (Supporting Information Fig. S2). \( CCA1::LUC \), unlike \( LHY::LUC \), shows very similar peak expression across temperatures. \( CAB2::LUC \), like that of \( CCA1 \) and \( LHY \), also shows a rapid and sharper increase in promoter activity at dawn for 27 °C data compared to 12 and 17 °C (Fig. 5 and Supporting Information Fig. S2). \( CCA1::LUC \), unlike \( LHY::LUC \), shows very similar peak expression across temperatures. \( CAB2::LUC \), like that of \( CCA1 \) and \( LHY \), also shows a rapid and sharper increase in promoter activity at dawn for 27 °C data compared to 12 and 17 °C. \( CAB2::LUC \) at 27 °C can be seen to have much higher promoter activity but due to dampening becomes lower than that of 17 °C by the end of the time-lapse experiment. For \( TOC1 \), a high peak of promoter activity was observed at dusk at 27 °C, which is consistent with the decreased levels of \( TOC1 \) repressors, \( LHY \) and \( CCA1 \). To further analyse this, we calculated the maximal phase of expression at 17 and 27 °C relative to that of 12 °C for each marker separately. Phase changes occurred for both \( TOC1 \) and \( LHY \) at 27 °C only, showing a 2 h phase advance (Supporting Information Fig. S3). No phase changes occurred across the temperature range of 12–27 °C for either \( CAB2 \) or \( CCA1 \). In addition to phase changes, a striking change in shape can be seen for \( TOC1 \) (Fig. 5) with a second peak occurring at the dark to light transition, which is enhanced as temperature increases. Interestingly, this second peak showed a ~6 h phase delay at 27 °C and a ~2 h phase delay at 17 °C when compared to 12 °C.

Together, these results are in agreement with a temperature-dependent change in the expression pattern of clock genes under diurnal conditions. They would also support the idea that a faster clock at higher temperatures may shift aspects of physiology and biochemistry earlier in the day.

**Variation in growth performance in high temperature**

Based on work from Dodd et al. (2005), which demonstrated that plants with an endogenous circadian period closely matching the natural day length outperform plants with a period mismatching against the external day length, we can predict that plants with well temperature-compensated clocks would perform best at higher temperatures. However, our observation, that the molecular clock period shortens at high temperature, is a feature conserved in all tested accessions of \( Arabidopsis \) and in \( B. rapa \) (Lou et al. 2011), and may point to the importance of a flexible clock capable of subtle adjustments in period. These changes could in turn coordinately regulate whole sets of genes subsequently affecting plant performance.

**Figure 4.** Effect of temperature on circadian leaf movement in \( Arabidopsis \) accessions. Plants were grown on MS agar under 12:12 L/D for 10 d before the transfer to 17 or 27 °C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, 17 °C (\( n = 20 \)); open squares, 27 °C (\( n = 20 \)). Accessions are presented in alphabetical order.
capacities. To check whether temperature-related changes in clock period could be of immediate importance to plant performance, we analysed the growth performance of each accession. For this performance study, we chose to measure total aerial biomass, as it is a strong overt indicator of plant growth performance (Dodd et al. 2005). Seedlings were germinated in compost and grown under 12:12 L/D at 22 °C for 12 d, before transferring to 12, 17 or 27 °C and continued 12:12 L/D cycles. The expression pattern for each marker has been graphed separately with plotted lines representing expression at 12 (black squares), 17 (empty circles) and 27 °C (grey triangles). The plots represent an average of at least three independently transformed lines. The experiment was repeated three times with the data shown here being a representative of the results gained.

We found that high temperature promoted growth in all accessions, but there was considerable variation in growth response between them (Fig. 6). At 17 °C, size and weight varied considerably between accessions. Therefore, weight gain between the 17 and 27 °C, rather than plant absolute weight, was used to represent performance data. The dry weight gain between 17 and 27 °C was accession dependent and varied from ~0.75 mg to >4 mg. Accessions Or-0, Ct-1, Phw-19 and Dog-5 had a small weight gain of less than 1 mg (Fig. 6), suggesting that their growth is less influenced by high temperature. In comparison, Eri, Col-0, Ler, Est, Wc-1 and Kyo were the accessions most responsive to 27 °C temperature, exhibiting more than 3 mg dry weight increase.

To investigate the relationship between plant performance and temperature-buffering capacity of the clock, we analysed plant dry weight gain and Q10 for each of our circadian markers (leaf movement, CCA1 and LHY) for each accession. We found no correlation between the temperature-buffering capacity of leaf movement period and dry weight (Fig. 7a,d). Furthermore, no correlation existed between

Figure 5. Temperature-dependent changes in the diurnal expression of clock-regulated genes CCA1, LHY, TOC1 and CAB2. Transgenic Col-0 seedlings carrying either CCA1::LUC, LHY::LUC, TOC1::LUC or CAB2::LUC reporter genes were entrained under 12:12 L/D cycles for 7 d, before transferring to 12, 17 or 27 °C and continued 12:12 L/D cycles. The expression pattern for each marker has been graphed separately with plotted lines representing expression at 12 (black squares), 17 (empty circles) and 27 °C (grey triangles). The plots represent an average of at least three independently transformed lines. The experiment was repeated three times with the data shown here being a representative of the results gained.
Figure 6. Natural variation in *Arabidopsis* growth performance at 27 °C. (a) Dry weight measured at 17 (dark bar) and 27 °C (light bar); (b) evaluation of dry weight gain at 27 °C. Seedlings were grown at 22 °C and 12:12 L/D for 12 d before transplanting to individual cell trays. After 2 additional days at 22 °C, all trays were moved to 17 or 27 °C. All plants were harvested after 14 d. Weight gain was calculated by subtracting the 17 °C weight mean from the 27 °C mean. For b, accessions are arranged from the smallest to the largest value.

Figure 7. Relationship between growth performance and temperature compensation of leaf movement (a, d) *CCA1* (b, e) and *LHY* (c, f). Seedlings were grown at 22 °C and 12:12 L/D for 12 d before transplanting to individual cell trays. After 2 additional days at 22 °C, all trays were moved to 17 or 27 °C. All plants were harvested after 14 d, dried in the 80 °C oven for 48 h and weighed. $1/Q_{10}$ was calculated from the equation $1/Q_{10} = (1/T_{27})/(1/T_{17})$, where $T_{17}$ and $T_{27}$ were period estimates at 17 and 27 °C, respectively. $1/Q_{10}$ is used for better visual representation of an increased oscillator speed, which is associated with a period shortening. $r$, correlation coefficient. (a, b and c) Weight gain was used to represent the weight increase associated with the 27 °C treatment and was calculated by subtracting the mean weight at 17 °C from the mean weight at 27 °C. (d, e and f) Relative weight gain was calculated by dividing weight gain between 17 and 27 °C by weight at 17 °C.
weight change and rhythm robustness of leaf movement (data not shown). This result was surprising, as leaf movement is the most buffered against temperature changes (Fig. 3). Interestingly, a clear trend was revealed between LHY temperature compensation and both raw growth performance data and data normalized to the 17 °C weight with correlation coefficients ($r$) of 0.575 ($P = 0.013$) and 0.514 ($P = 0.029$), respectively (Fig. 7c,f). The interaction between weight and CCA1 temperature compensation was also significant when using the raw performance data ($r = 0.467; P = 0.051$), although this becomes slightly non-significant when normalized to 17 °C weight ($r = 0.350; P = 0.154$; Fig. 7b,e). These data imply that accessions with circadian clocks that are poorly buffered against temperature, that is, having greater period difference between 17 and 27 °C, gain more weight at high temperature, while growth of those with well-buffered clocks is compromised. Therefore, it appears that a flexible endogenous clock might be more advantageous than a perfectly buffered clock.

### Sequence variation of core clock genes across accessions

It is entirely possible that evolution has selected specific components within the clock to achieve optimum temperature-dependent period changes that are best suited to the environment. In Neurospora crassa, natural variation in circadian period and temperature compensation has been linked to the length of the activation domain of the WC-1 protein, more specifically, to the number of simple sequence repeats (SSRs) present in the NpolyQ (amino-terminal polyglutamine domain) region (Michael et al. 2007). It is possible that a similar molecular source causes phenotypic circadian variation in temperature compensation in Arabidopsis accessions.

To try and identify such molecular components underlying phenotypic circadian variation in temperature compensation, we have utilized data generated from the 1001 genome project. We downloaded sequence data for all the key clock genes PRR9, CCA1, ELF4, GI, LHY, LUX, TOC1, ELF3 and PRR7, for 14 of our phenotyped accessions. The sequences included the complete coding region, together with 1 kb of upstream sequence. The genes were aligned and used to make phylogenetic trees (Supporting Information Fig. S4). From this analysis, clear clusters of accessions could be seen for different clock genes. This was most pronounced for PRR7 where the accessions could be divided up into two clear haplotypes. These two haplotypes have been previously suggested as responsible for a period QTL between Ler and Col-0 (Michael et al. 2003a). However, we failed to find a correlation between clustered genotypes with clusters of similar temperature-dependent clock phenotypes.

### DISCUSSION

In this study, we show natural variation in temperature buffering of the circadian clock and a clear trend in period shortening of the clock in response to increasing temperature. We demonstrate that temperature-dependent period shortening in free-running conditions may have consequences for the temperature-dependent changes in expression patterns we observe for clock genes in diurnal cycles. Finally, we demonstrate a correlation between decreased temperature-buffering capacity and enhanced performance, suggesting that a shorter period at higher temperature may confer a performance advantage.

Natural variation in temperature compensation of leaf movement rhythms has previously been described (Edwards et al. 2005). Here, we identified variation in the temperature response of two key clock genes, CCA1 and LHY. While temperature caused a more uniform effect on the circadian period of CCA1 across accessions, temperature effects on LHY period were more accession specific. Therefore, our study has uncovered temperature-dependent differences in the coupling of these two key clock genes, suggesting that at higher temperatures in some accessions, these two genes may be driven by different oscillators. Similar period differences in a single plant have been reported for CAB2 and PHYB (phytochrome B) rhythms even though both seem to be regulated by similar circadian clock control mechanisms (Hall et al. 2002). In addition, cytosolic free calcium ($Ca^{2+}$) and CAB2:LUC have different free-running periods under constant red light conditions (Sai & Johnson 1999). Furthermore, CAT3 (Catalase 3) and CAB2 also have different periods after entrainment to different temperature cycles, indicating that at least two oscillators with different temperature sensitivities are present within the plant (Michael, Salome & McClung 2003b). It is possible that this uncoupling may confer further temperature-dependent flexibility on the expression patterns of circadian-regulated genes; however, at present, we have no information on whether uncoupling persists under diurnal cycles.

It is possible that multiple oscillators uncouple in response to stress. Due to variation in the geographical locations between Arabidopsis accessions and the lack of associated information on collection site environments or collection times, it is difficult to predict the degree of stress different accessions have undergone during natural selection (Hoffmann 2002). While 17 °C is considered to be ‘normal’ for common laboratory strains, it might be stressful for some ‘wild’ Arabidopsis lines, therefore, resulting in an early uncoupling of circadian oscillators/loops explaining differentiation between CCA1 and LHY in some accessions (Table 2). Apart from temperature-induced period shortening, the robustness of the CCA1 and LHY rhythmicity was also affected, with LHY being affected more severely than CCA1. Gould et al. (2006) demonstrated that functional LHY was more important for Arabidopsis at higher temperature, while CCA1 was more important at lower temperature. Despite this temperature-dependent differentiation, expression of LHY was highly down-regulated by the 27 °C treatment in comparison to 17 °C. Expression of CCA1, on the other hand, remained unaffected. Overall, it is intriguing how the importance of LHY transcription increases with its increase in temperature sensitivity (Gould et al. 2006).
Our analysis of sequence variation of key clock genes across 14 accessions identified clustering of accessions with specific genotypes (Supporting Information Fig S4); however, these failed to match with a similar cluster of temperature-dependent clock phenotypes. It is entirely possible that the genetic variation responsible for the phenotypic variation lies outside the limited set of genes investigated. Many recent studies (Gould et al. 2006; Portoles & Mas 2010; Salome et al. 2010) describe clock components potentially involved in temperature compensation. However, it is more likely to be a feature of the whole network or subset of components rather than a single gene or mechanism (Gould et al. 2013). Therefore, it may be difficult in this single-gene analysis approach to identify the molecular sources of variation. Finally, here we have studied variation at the whole gene level. It is possible that at this level single point mutations that are responsible for the phenotype differences have been masked. While this is feasible, what we have found in our analysis is that different accessions tend to have several linked polymorphisms so while a single SNP maybe the causative SNP, it would be associated with a set of linked SNPs. These linked regions would be sufficient to cluster accessions in our trees.

A robust circadian clock with an oscillator period resonant with that of the external L/D cycle enhances growth and survival of Arabidopsis plants (Dodd et al. 2005). Therefore, initially, we had hypothesized that accessions with a well-buffered clock would perform better when grown under elevated temperatures. Our findings were contrary to this assumption. Accessions with the least temperature-dependent period differences for rhythms of CCA1 and LHY showed the smallest increases in growth with raising temperature. This was not observed for the leaf movement rhythm, even though it was thought to be more temperature compensated. This suggests that the relationship between the temperature, circadian clock and performance is complex. Indeed, several studies have shown the existence of an important link between the circadian clock and plant performance, but this link turns out to be not straightforward. For example, Graf et al. (2010) observed that a short period cca1::lhy double mutant performed better under 20 h rather than 24 h LD cycle. However, while a short-period toc1 mutant performs better under 20 h rather than 28 h, its best performance was still at 24 h (Dodd et al. 2005; Graf et al. 2010). Correlation between weight gain and period is stronger for LHY::LUC than for CCA1::LUC. Of course, a change in gene transcription does not necessarily directly translate into changes in protein function; however, it is tempting to speculate that transcription of LHY has a greater involvement in plant growth regulation at higher temperatures. In addition, temperature influences alternative splicing of CCA1 and LHY, which might be important in growth regulation under changing environmental conditions (James et al. 2012). Effects of temperature on transcript and protein stability and function should also be examined.

It would be of great interest to further investigate the effects of the temperature-buffered clock on other aspects of plant performance, for example, chlorophyll content, seed quantity and viability, etc. Flowering time is another aspect of plant physiology that is affected by the circadian clock and temperature, and their relationship across accessions would be valuable to examine. Flowering data was not collected in this study, but it was apparent that flowering time varied between accessions. For the growth performance experiment, all plants were harvested before any accessions bolted; however, it is possible that the growth rate of some plants could have been affected by the vegetative-to-reproductive transition. Nevertheless, our data provides preliminary insights into how the circadian clock could be a major contributor to plant fitness and possibly survival in response to temperature fluctuations in the environment.

An intriguing question is, ‘why is the clock not perfectly buffered against temperature?’, and in fact, ‘why could having a short period at higher temperatures enhance growth?’ One possibility is that the temperature-dependent shortening of period we observe and the altered expression in diurnal cycles may act as an escape mechanism. This would allow the plant to temporally shift temperature-sensitive activities to avoid the warmest and most damaging parts of the day. This idea is further supported by the fact that the desert succulent plant Kalanchoe fedtschenkoi also has a clock that oscillates with a short period (Anderson & Wilkins 1989), although it is possible that K. fedtschenkoi is not representative of all desert plants. Similarly, the uncoupling mechanism we observe may be a method of allowing temporal shifts for some biological processes while maintaining the coupling of others to a well temperature-buffered 24 h oscillator.

ACKNOWLEDGMENTS

We would like to acknowledge funding from a Marie Curie Early Stage Training project MEST-CT-2005–020526 for J.K. and the BBSRC for A.H. and P.D.G. (BB/H022333/1, BB/F005318/1). We would like to thank the European Arabidopsis Stock Centre (NASC) for supplying the Arabidopsis accessions used in this project and Lázlo Kozma Bognár for the LHY::LUC and CCA1::LUC constructs. We would also like to thank Antony Dodd and Paul Fogg for critically reading the manuscript.

REFERENCES

Alabadi D., Oyama T., Yanovsky M., Harmon F., Mas P. & Kay S. (2001) Reciprocal regulation between TOCI and LHY/CCA1 within the Arabidopsis circadian clock. Science 293, 880–883.
Albrecht U. (2002) Functional genomics of sleep and circadian rhythm: invited review: regulation of mammalian circadian clock genes. Journal of Applied Physiology 92, 1348–1355.
Anderson C.M. & Wilkins M.B. (1989) Period and phase control by temperature in the circadian rhythm of carbon dioxide fixation in illuminated leaves of Bryophyllum fedtschenkoi. Planta 177, 456–469.
Anwer M.U. & Davis S.J. (2013) An overview of natural variation studies in the Arabidopsis thaliana circadian clock. Seminars. Cell Developmental Biology 24, 422–429.
Clough S.J. & Bent A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant Journal 16, 735–743.

© 2013 The Authors. Plant, Cell & Environment published by John Wiley & Sons Ltd, 37, 327–340
Dunlap J.C. (1999) Molecular bases for circadian clocks. *Plant Methods* 5, 3–10.

Desfeux C., Clough S.J. & Bent A.F. (2000) Female reproductive tissues are the primary target of Agrobacterium-mediated transformation by the Arabidopsis floral-dip method. *Plant Physiology* 123, 895–904.

Dodd A., Salathia N., Hall A., Kevei E., Toth R., Nagy F., Hibberd J., Millar A. & Webb A. (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309, 630–633.

Dunlap J.C. (1999) Molecular bases for circadian clocks. *Cell* 96, 271–290.

Edwards K.D., Lynn J.R., Gyula P., Nagy F. & Millar A.J. (2005) Natural allelic variation in the temperature-compensation mechanisms of the Arabidopsis thaliana circadian clock. *Genetics* 170, 387–400.

Gardner M.J., Hubbard K.E., Hotta C.T., Dodd A.N. & Webb A.A.R. (2006) How plants tell the time. *Biochemical Journal* 397, 15–24.

Gendron J.M., Pruneda-Paz JL., Doherty CJ., Gross A.M., Kang S.E. & Kay SA. (2012) Arabidopsis circadian clock protein, TOC1, is a DNA-binding transcription factor. *Proceedings of the National Academy of Sciences of the United States of America* 109, 3167–3172.

Gould P.D., Locke J.C., Larue C., et al. (2006) The molecular basis of temperature compensation in the Arabidopsis circadian clock. *The Plant Cell* 18, 1177–1187.

Gould P.D., Ugarte N., Domijan M., et al. (2013) Network balance via CRY signalling controls the Arabidopsis circadian clock over ambient temperatures. *Molecular Systems Biology* 9, 650.

Graf A., Schlereth A., Stitt M. & Smith A.M. (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. *Proceedings of the National Academy of Sciences of the United States of America* 107, 9458–9463.

Green R.M., Tingay S., Wang Z.Y. & Tobin E.M. (2002) Circadian rhythms confer a higher level of fitness to Arabidopsis plants. *Plant Physiology* 129, 576–584.

Hall A., Kozma-Bognar L., Bastow R.M., Nagy F. & Millar A.J. (2002) Distinct regulation of CAB2 and PHYB gene expression by similar circadian clocks. *The Plant Journal: For Cell and Molecular Biology* 32, 529–537.

Harmer S.L. (2009) The circadian system in higher plants. *Annual Review of Plant Biology* 60, 357–377.

Highkin H.R. & Hanson J.B. (1954) Possible interactions between light-dark cycles and endogenous daily rhythms on the growth of tomato plants. *Plant Physiology* 29, 301–302.

Hoffmann M.H. (2002) Biogeography of Arabidopsis thaliana (L.) Heynh. *Brassicaceae*. *Journal of Biogeography* 29, 125–134.

James A.B., Syed N.H., Bordage S., Marshall J., Nimmo G.A., Jenkins G.I., Alabouvette C., Hotta C.T., Dodd A.N. & Webb A.A.R. (2006) A rice circadian clock reporter of circadian gene expression in cyanobacteria. *Molecular Systems Biology* 2, 509.

Johnson C.H. (1993) Circadian rhythms in prokaryotes – luciferase as a reporter of circadian clock genes. *Bioluminescence and Chemiluminescence in Microorganisms*. 125–134.

Kondo T., Strayer C.A., Kulkarni R.D., Taylor W., Ishiura M., Golden S.S. & Golden L. (2002) Circadian rhythms in the cyanobacterium Synechococcus sp. PCC 7942 are controlled by an endogenous circadian clock. *Proceedings of the National Academy of Sciences* 99, 12372–12377.

Kopchik A., Fernandez A.P., Edwards K.D., Soubon M.H., Halliday K.J. & Millar A.J. (2012) The clock gene circuit in Arabidopsis includes a repressor with additional feedback loops. *Molecular Systems Biology* 8, 574.

Portoles S. & Mas P. (2010) The functional interplay between protein kinase CK2 and CCA1 transcriptional activity is essential for clock temperature compensation in Arabidopsis. *PLoS Genetics* 6, e1000120.

Sai J. & Johnson C.H. (1999) Different circadian oscillators control Ca²⁺ fluxes and Lhcb gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 96, 11659–11663.

Salome P.A., Weigel D. & McCpng C.R. (2010) The role of the Arabidopsis morning loop components CCA1, LHY, PRR7, and PRR9 in temperature compensation. *The Plant Cell* 22, 3630–3661.

Swarup K., Alonso-Blanco C., Lynn J.R., Michaels S.D., Amasino R.M., Koornneef M. & Millar A.J. (1999) Natural allelic variation identifies new genes in the Arabidopsis circadian system. *Plant Journal* 20, 67–77.

Tsondor S.J., Scott C., Bouchaz L., Liss T.R., Brodsky J.L. & Vierling E. (2008) Heat shock protein 101 effects in *A. thaliana*: genetic variation, fitness and pleiotropy in controlled temperature conditions. *Molecular Ecology* 17, 1614–1626.

Zinn K.E., Tunc-Ozdemir M. & Harper J.F. (2010) Temperature stress and plant sexual reproduction: uncovering the weakest links. *Journal of Experimental Botany* 61, 1959–1968.

Received 8 May 2012; received in revised form 29 May 2013; accepted for publication 30 May 2013

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Normalized average traces for CCA1::LUC (squares) and LHY::LUC (triangles) activity at 17 °C (solid) and 27 °C (empty). Plants were grown on MS agar under 12:12 L/D for 10 days before the transfer to 17 °C or 27 °C and continuous light, at which CCA1::LUC and LHY::LUC rhythms were assessed. First 24 h after the transfer to continuous light were not included in period analysis, n = 8 for all accessions except Ws-2, where n = 2.

**Figure S2.** This figure is the sample data as in figure 5 but non-normalised. Temperature dependent changes in the diurnal expression of clock regulated genes CCA1, LHY, TOC1 and CAB2. Transgenic Col-0 seedlings carrying either CCA1::LUC, LHY::LUC, TOC1::LUC or CAB2::LUC reporter genes were entrained under 12:12 L/D cycles for 7 d, before transferring to 12, 17, or 27 °C and continued 12:12 L/D cycles. The expression pattern for each marker has been graphed separately with plotted lines representing
expression at 12 °C (black squares), 17 °C (empty circles) and 27 °C (grey triangles). The plots represent an average of at least 3 independently transformed lines. The experiment was repeated 3 times with the data shown here being a representative of the results gained.

**Figure S3.** The phase of maximal expression in 12h L/12h D cycles at 17 and 27 °C was compared to 12 °C for each marker separately. A change in phase occurs at 27 °C for both TOC1 and LHY markers with a ~2 h phase advance. No phase changes occurred across the temperature range of 12–27 °C for both CAB2 and CCA1.

**Figure S4.** Phylogenetic analysis of key circadian clock genes. Gene sequences (1KB upstream plus coding region) were downloaded from the Arabidopsis 1001 genome browser (http://signal.salk.edu/atg1001/index.php) for 14 of the phenotyped accessions. Sequences were aligned using MUSCLE. Plotted in the figure are phylogenetic tree for each of the genes, drawn using the Jukes Cantor model and the Neighbour joining tree-building method. A. unrooted tree for 14 accession and 9 key clock genes. B. Tree for 14 accession and rooted with sequence from Arabidopsis lyrata for CCA1 and PRR7.