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Circadian rhythms are associated with variation in photosystem II function and photoprotective mechanisms

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Abstract. The circadian clock regulates many aspects of leaf gas supply and biochemical demand for CO_2, and is hypothesized to improve plant performance. Yet the extent to which the clock may regulate the efficiency of photosystem II (PSII) and photoprotective mechanisms such as heat dissipation remains largely unexplored. Based on measurements of chlorophyll *a* fluorescence, we estimated the maximum efficiency of photosystem II in light (*Fv'/Fm'*) and heat dissipation by non-photochemical quenching (*NPQ*). We further dissected total *NPQ* into its main components, *qE* (pH-dependent quenching), *qT* (state-transition quenching) and *qI* (quenching related to photoinhibition), in clock mutant genotypes of *Arabidopsis thaliana*, the cognate wild-type genotypes, and a panel of recombinant inbred lines (RILs) expressing quantitative variation in clock period. Compared to mutants with altered clock function, we observed that wild-type genotypes with clock period lengths of approximately 24 hr had both higher levels of *Fv'/Fm'*, indicative of improved PSII function, and reduced *NPQ*, suggestive of lower stress on PSII light harvesting complexes. In the RILs, genetic variances were significant for *Fv'/Fm'* and all three components of *NPQ*, with *qE* explaining the greatest proportion of *NPQ*. Bivariate tests of association and structural equation models of hierarchical trait relationships showed that quantitative clock variation was empirically associated with *Fv'/Fm'* and *NPQ*, with *qE* mediating the relationship with gas exchange. The results demonstrate significant segregating variation for all photoprotective components, and suggest the adaptive significance of the clock may partly derive from its regulation of the light reactions of photosynthesis and of photoprotective mechanisms.

Key words: *Arabidopsis thaliana,* circadian rhythms, chlorophyll *a* fluorescence, maximum efficiency of PSII, non-photochemical quenching

Introduction

The circadian clock is a time-keeping mechanism that enables organisms to adaptively match many transcriptomic, physiological, developmental, and biochemical processes to natural diurnal cycles (McClung *et al*., 2013; Yerushalmi *et al*., 2009; Sanchez *et al*., 2016; Resco de Dios and Gessler, 2017). By comparing the phenotypes of wild-type plants to mutant genotypes with altered clock function, several studies have demonstrated that diverse ecophysiological traits (e.g., total CO_2 assimilation rates and sugar status) are affected by the circadian clock (Dodd *et al*., 2005; Graf *et al*., 2010). More specifically, circadian rhythms that are closer to 24 hours and resonate with environmental cycles likely optimize the diurnal timing of gas exchange (Dodd *et al*., 2005). Transcriptomic studies on representative *Arabidopsis* genotypes also indicate that key gas-exchange genes are regulated on a diel basis (Dodd *et al*., 2014; Pilgrim & McClung, 1993). Further, quantitative variation in the circadian clock is associated with gas-exchange in segregating progenies (Edwards *et al*., 2011; Lou *et al*., 2011) and in crop types of *Brassica rapa* (Yarkhunova *et al*., 2016) as well as with timing of gas-exchange responses to drought (Greenham *et al*., 2017). Thus, the circadian clock emerges as an important regulator of gas-exchange. Yet, its influence on the biophysical activity of both photosystems remains poorly characterized, leaving unresolved the mechanistic connection between the circadian clock and leaf level gas-exchange as well as photoprotection (Greenham & McClung, 2015; Guadagno *et al*., 2018).

Sunlight serves as the energy source for photosynthesis, and higher light intensities typically correlate with increases in photosynthetic rates (*A*) (Björkman &

65 Demmig-Adams, 1995; McDonald, 2003). Further, the efficiency of photosystem II
66 (PSII) in utilizing light energy (Fv'/Fm') correlates with gas-exchange rates and plant
67 performance under various experimental conditions at a given light level (Maxwell &
68 Johnson, 2000). However, the absorbed light energy may exceed the demand for energy
69 and the reducing capacity of the light-independent reactions of photosynthesis,
70 potentially leading to photodamage through formation of reactive oxygen species (ROS).
71 In response to light stress, plants have evolved several photoprotective mechanisms. A
72 large number of enzymes take part in scavenging activities (Asada, 2006; Das &
73 Roychoudhury, 2014); some carotenoids have been shown to be highly efficient in
74 scrubbing excited chlorophyll molecules (Bassi & Caffarri, 2000), and ascorbate is also
75 an efficient antioxidant in various organisms (Fukumura *et al*., 2012). However, when
76 excitation energy exceeds demand, the first line of defense to avoid damage to PSII is
77 heat dissipation. Thermal dissipation is a protective strategy to reduce photoinhibition,
78 and is ubiquitous to photosynthetic organisms (Müller *et al*., 2001). This mechanism
79 competes with photochemistry and chlorophyll *a* fluorescence for the use of excitation
80 energy (Baker, 2008), and it is commonly referred to as non-photochemical quenching of
81 chlorophyll *a* fluorescence (*NPQ*).
82 *NPQ* comprises at least three major components: *qE* (pH-dependent quenching),
83 *qT* (state-transition quenching) and *qI* (quenching related to photoinhibition). The onset
84 of *qE* occurs quickly, within seconds to a few minutes, and is triggered through the
85 synergistic action of thylakoid lumen pH and the formation of an energy quenching
86 complex between the protein PsbS and the pool of xanthophyll and zeaxanthin (Horton *et*
87 *al*., 2000; Li *et al*., 2002). The *qT* component can occur following 2-15 minutes of

88 illumination and reflects the balance of excitation between the two photosystems, which
89 depends upon reversible photophosphorylation activity and ensuing relocation of light
90 harvesting complexes (Niyogi, 2000). *qI* has slow relaxation kinetics and is related
91 directly to photoinhibition, including down-regulation and complete deactivation of PSII
92 (Li *et al*., 2002).

93 In the past two decades, the development of pulse amplitude modulated (PAM)
94 fluorometry has provided a sensitive and non-destructive method to estimate the
95 efficiency of PSII and the importance of *NPQ* and the variability of each component in
96 different environmental conditions (Baker, 2008; Schreiber, 2004). Among several
97 applications, the PAM method has made it possible to partition variance among
98 environmental and genetic sources. Prior studies have focused on partitioning sources of
99 variance in total *NPQ* (Fujiwara *et al*., 2014; Jung & Niyogi, 2009; Kasajima *et al*.,
100 2011; van Rooijen *et al*., 2015) and in PSII photoinhibition (Jansen *et al*., 2010). Genetic
101 variances for total *NPQ* were highly significant in four *A. thaliana* accessions across an
102 extensive range of incident light (varying from 100 to 1800 μmol photons $m^{-2}s^{-1}$; (Jung &
103 Niyogi, 2009). However, the magnitude of genetic variances of all individual components
104 of *NPQ* have not been estimated, although such knowledge is important to understanding
105 possible regulatory paths and ultimately to breeding opportunities for crop improvement.

106 Light availability and light stress vary in predictable ways over the course of the
107 day. Quantitative clock variation is correspondingly associated with gas-exchange in
108 various species under field and controlled environmental conditions (Burstin *et al*., 2007;
109 de Dios *et al*., 2016; Edwards *et al*., 2012; Edwards *et al*., 2011; Yarkhunova *et al*.,
110 2016), and might contribute to the regulation of thermal dissipation of excess energy.

Further, although thermal dissipation is a photoprotective mechanism, it is metabolically regulated and impacts the operational state of photosynthesis (Murchie & Harbinson, 2014), again consistent with the hypothesis that *NPQ* might be clock regulated.

Here, we first compared the maximum efficiency of PSII in light (*Fv'/Fm'*) and *NPQ* between wild-type genotypes of *Arabidopsis thaliana* and mutants with altered clock function to empirically test for a possible role of the circadian clock in PSII function and photoprotection. We then used recombinant inbred lines (RILs) that vary in circadian periodicity to characterize the expression of genetic variation in leaf gas exchange, chlorophyll *a* fluorescence traits, and *NPQ* across environments with high *vs.* low light intensity. Finally, we used structural equation modeling to investigate hypothesized causal relationships between quantitative variation in circadian rhythms, leaf gas exchange, *NPQ*, and the components of *NPQ*.

Materials and Methods

Plant material and growth

We first compared *Fv'/Fm'* and total *NPQ* between mutant genotypes with altered clock function and the cognate wild-type plants, in order to test the relationship between clock (mis)function and efficiency of PSII function and photoprotection. We included replicates harboring alleles of the clock mutant genotype, *zeitlupe* (*ztl-24, ztl-25*); (Kevei *et al*., 2006), that express a long clock period (28 hr) phenotype, the clock mutant, *timing of cab expression 1* (*toc1-21*) (Ding *et al*., 2007; Fujiwara *et al*., 2008) that express a

134 short clock period (20 hr), and the cognate, Ws-2, wild-type genotype in which these
135 mutations reside.

136 Seeds of both mutant and wild type genotypes were placed in microcentrifuge
137 tubes stratified in water at 4°C for 1 week. Seeds were then planted into 6 × 6 × 9 cm
138 plastic pots filled with Sunshine #5 potting mix (Sunshine Redi-Earth Professional
139 Growing Mix, Sun Gro Horticulture, Bellevue, WA). Pots were placed in Percival PGC-
140 9/2 growth chambers (Percival Scientific, Perry, Indiana, USA) with the following
141 conditions: photoperiod 10/14 hours (light/dark), temperatures of 22 ± 1 °C during the
142 daytime and 19 ± 1 °C during nighttime, and PPFD = 350 μmol photons m^{-2} s^{-1}. Short
143 days were used to allow for greater growth before the onset of flowering. Measurements
144 of *Fv'/Fm'* and *NPQ* were taken at the ambient light level of 350 μmol photons m^{-2} s^{-1} on
145 at least seven replicates per genotype using a portable PAR-FluorPen FP 100-MAX-LM
146 fluorometer (Photon System Instruments, Brno, Czech Republic).

147 To characterize genetic and environmental sources of variation in *Fv'/Fm',*
148 components of *NPQ,* and associations between these two traits and clock period, we used
149 recombinant inbred lines (RILs) of *Arabidopsis thaliana (L.)* Heynh. (*Brassicaceae*). The
150 RILs were developed from a cross between Ler (Landsberg *erecta*, Germany) and Ws-2
151 (Wassilewskaja, Belarus), in which the Ws-2 parent harbors the reporter gene
152 *LUCIFERASE (LUC)* linked to the promoter of *COLD-CIRCADIAN RHYTHM–RNA*
153 *BINDING 2* (*CCR2*), allowing for quantification of circadian parameters (Millar, Short,
154 Chua & Kay, 1992). Details of the crossing design are provided in Boikoglou & Davis
155 (2009) and Rubin *et al* (2017). In brief, the two parents were crossed to create a

heterozygous F_1. The F_1 was then backcrossed to the maternal parent, and the resulting BC_1F_2 genotypes were selfed to the BC_1F_6 generation through single seed descent.

An initial experiment quantifying *Fv'/Fm'* associations with clock period was conducted using 32 lines, following the same planting protocol and growth conditions as the mutants. Due to the time-consuming nature of *NPQ* relaxation curve measurements and limited space in the growth chambers, eleven RILs (8-10 replicates per RIL) were chosen at random to conduct the leaf chlorophyll *a* fluorescence measurements and to dissect the components of *NPQ*.

Circadian measures

For circadian measures, seeds of each RIL were surface-sterilized and cold-stratified. Six to eight replicates of each RIL were planted into white 96-well microliter plates containing Murashige and Skoog mineral plant growth media supplemented with 30g/L sucrose. Plates were then moved to the growth chambers with the following conditions: 10/14 hours (light/dark) photoperiod, temperature of 22 ± 1 °C and relative humidity of 50 ± 1 % for five days, a period of time sufficient for clock entrainment. After entrainment, 20μl of a 100 mM D-luciferin monopotassium salt and 0.01% Triton X-100 solution was added to each well, and plates were resealed and placed under an ORCA-II ER digital camera (Hamamatsu Photonics C4742-98-24ER). Circadian parameters were estimated from bioluminescence using fast Fourier transform nonlinear least-square analysis (FFT-NLLS) (Hicks *et al*., 1996).

Leaf gas-exchange and chlorophyll fluorescence measurements

Leaf gas-exchange measurements, including photosynthetic rate (*A*), stomatal conductance (g_s), and chlorophyll *a* fluorescence emissions, were measured simultaneously using a leaf chamber fluorometer LICOR LI-6400-40 (Open System Vers. 4.0, Li-Cor, Inc., Lincoln, NE). Measurements were taken from a fully developed rosette leaf at least 1 h after subjective dawn under the following chamber conditions: PPFD= 500 (low light, LL) or 1500 (high light, HL) μmol m^{-2} s^{-1}, flow rate= 300 m^{-2} s^{-1}, ref [CO_2] = 400 μmol m^{-2} s^{-1}, T_{leaf}= 22°C and VPD_L (Vapor pressure deficit based on leaf temp, kPa) was kept between 1.3-1.7 kPa, fan mode set on FAST (Long & Bernacchi, 2003). After a dark acclimation period (30 min), the maximum fluorescence in darkness (F_m) was determined by applying a saturating pulse (0.8 s) with intensity of ~5000 μmol photons m^{-2} s^{-1}. The leaves were then exposed for 10 min to different actinic light levels to obtain the maximum fluorescence in light conditions, F_m'. Calculations of Fo' used the equation from Oxborough and Baker (1997), $Fo'=Fo/(Fv/Fm+Fo/Fm')$. After induction of *NPQ*, recovery of the fluorescence signal was monitored in darkness for 40 min, through the application of seven saturating pulses (0.8 s; intensity of ~5000 μmol photons m^{-2} s^{-1}) at different times (2, 5, 10, 15, 20, 30, 40 min). *NPQ* data were expressed as $NPQ=(F_m-F_m')/F_m'$ (Bilger & Schreiber, 1987), and the three *NPQ* components (*qE*, *qT* and *qI*) were quantified following a modified method of Walters and Horton (Walters & Horton, 1990, Walters & Horton, 1991). For each recorded fluorescence curve and each measured leaf, *NPQ* data were reported in a semi-logarithmic plot versus recovery time. The components of *NPQ* were calculated by linear regression of three exponential

202 decays. The half-times for each component were reported as qI = A, qT = (B – A), qE =
203 (C – B), with A, B and C intercepts on the y axis (D'Ambrosio *et al*., 2008).
204
205 ***Data analysis***
206
207 *Statistical approach and data treatments*
208
209 All analyses were conducted in R version 3.2.4 (Team, 2014), http://www.r-project.org.
210 Analysis of variance (ANOVA) was used to test for differences in *Fv'/Fm'* and total *NPQ*
211 between wild-type and clock mutant genotypes in the first experiment. ANOVA was also
212 used to test the influence of light treatments and genotypic effect on physiological traits
213 (including circadian period, *Fv'/Fm'*, total *NPQ, A,* g_s*, qE, qT, qI*) measured in the RILs
214 ('lm' and 'anova' functions of R). Further, we estimated the fold difference in *NPQ* or its
215 components by dividing the trait value in one light treatment by its value in the other
216 treatment (low light / high light treatment). Principal components analysis (PCA) was
217 performed using the 'prcomp' procedure in R, and scores were tested for the effect of
218 genotype.
219 We were further interested in testing the relative contribution of individual
220 physiological traits and circadian period to the expression of A_{max}. First, we determined
221 how clusters of traits related to genetic variation in the RILs using Principal Components
222 Analysis (PCA) as an approach to address collinearity between fluorescence variables.
223 Second, to quantify hypothesized causal relationships between traits, we used structural
224 equation modeling with observed variables. We developed an initial (saturated) model

225 based on observed bivariate correlations and known relationships among physiological
226 traits and between circadian and physiological traits. The fit of alternative structural
227 equation models to the observed data was tested with the sem() function of the 'lavaan'
228 package (Rosseel, 2012) in R version 3.2.4 (Team, 2014). To identify a model with good
229 fit, a proposed model was evaluated through Confirmatory Factor Analysis within the
230 lavaan package and the fit indices that rank parsimony (Akaike's Information Criterion;
231 AIC). If the fit criteria (described below) were not met for the proposed model, then
232 modification indices were used to adjust the model; specifically, variables were excluded
233 from the model with the highest AIC, and fit indices for the reduced model were again
234 evaluated. Model fit was assessed with a chi-square test, root mean square error of
235 approximation (RMSEA), and comparative fit index (CFI). Chi-square values associated
236 with a P-value > 0.05 and a RMSEA <0.05 and CFI > 0.95 indicate a good fit of the
237 model to the data (Kline, 2015).
238 Once the model with the best fit was identified, structural equation modeling was
239 used to partition variation in a response variable among multiple predictor variables.
240 Specifically, the multivariate regression model that is the basis for structural equation
241 modeling statistically accounts for variation in multiple predictor variables (in this case,
242 traits) simultaneously and tests their relationship to a response variable. We were
243 interested in the hierarchical relationships among measured traits (e.g., circadian period,
244 gas-exchange traits, *NPQ*). This approach reveals the extent to which a given trait
245 directly *vs*. indirectly affects the response variable (e.g., circadian period could affect A_{max}
246 directly or act indirectly through *NPQ*) (e.g., Fournier-Level *et al*, 2013).
247

Results

To test for a clock effect on chlorophyll fluorescence, we compared *Fv'/Fm'* and total *NPQ* between wild-type plants that express a circadian period near 24 hrs to clock mutant genotypes with short 20-hr (*toc1*) or long 28-hr (*ztl*) circadian cycles (Fig. 1). Analysis of variance revealed a significant genotype effect on maximum efficiency of PSII in light (*Fv'/Fm'*) (Table 1a). Specifically, wild type Ws-2 plants had higher values of *Fv'/Fm'* compared to short and long circadian period mutants, indicating that light absorbed by PSII is converted more efficiently to photochemistry in the wild-type plants (Fig. 1a). Furthermore, ANOVA showed that circadian clock mutants had higher values of *NPQ* than the wild type (Fig. 1b), indicating potentially greater light stress and the need for higher thermal dissipation in the mutant genotypes even under the comparatively low light treatment conditions. In sum, the results suggest that significant deviations (±4 h) from a wild-type circadian period of approximately 24 hrs may lead to reduced PSII efficiency and to a surplus of excitation energy for PSII.

Genetic variation in RILs, light treatment effects, and bivariate correlations

We first surveyed circadian period and other physiological parameters, including photosynthetic rate (*A*), stomatal conductance (g_s) and maximum efficiency of PSII in light (*Fv'/Fm'*) in 32 RILs. Analysis of variance showed significant variation among RILs in circadian period and all physiological traits (Table 1). Among the RILs, we observed a significant association between *Fv'/Fm'* and circadian period, such that RILs with

circadian cycles closer to 24 hrs had higher quantum yield of PSII (Fig. 2a).

We then chose a subset of eleven genotypes to estimate genetic and environmental variances in the underlying fluorescence and non-photochemical quenching parameters under our two experimental light conditions (low light, LL, 500 μmol photons m^{-2} s^{-1} and high light, HL, 1500 μmol photons m^{-2} s^{-1}) and to further explore the relationship between the circadian clock and chlorophyll *a* fluorescence. We observed significant light treatment effects for *A*, *Fm'*, F_v'/F_m', *NPQ*, *qE*, *qT*, and *qI* ($p<0.001$; Table 1). As expected, *A* decreased in low light conditions, while F_v'/F_m' decreased in response to the high light conditions (Table 1c). *NPQ* typically rises with increasing light intensity and light stress, and we correspondingly observed a significant increase in total *NPQ* under the HL relative to LL treatment ($p<0.0001$; Fig. 3a). The partitioning of individual components of *NPQ* also varied across light treatments (Fig. 3b, c). Within total *NPQ*, *qE* and *qT* were higher on average in the LL treatment, while *qI* was higher in the HL treatment (Fig. 3b, c). Overall, in both treatments *qE* was the primary determinant of total *NPQ* (Fig. 3b, c).

The subset of 11 RILs also differed significantly in the expression of all measured physiological parameters (Table 1; Fig. 3). Specifically, *A*, g_s, *Fm'*, F_v'/F_m', *NPQ*, *qE*, *qT* and *qI* showed a significant genotype effect ($p<0.001$; Table 1). Total *NPQ* differed by 60% between RILs with the highest *vs*. lowest values under HL and 59% under LL (Fig. 3a). Using LL for further comparison of the *NPQ* components, *qI* and *qT* differed by more than 100% between RILs with the highest *vs*. lowest values of these two traits; in particular, *qT* differed by 166% between RIL113 and Ws-2 under the LL treatment, while *qI* differed by 175% between RIL36 and RIL136. Differences among RILs were less

pronounced for *qE*, which varied by at most 12% among RILs in LL (Fig. 3b).

To empirically assess relationships among physiological traits, we tested for significant bivariate correlations. As expected, *A* was correlated positively with g_s. *A* was also positively correlated with F_v'/F_m' and with other fluorescence parameters (*Fm'*, *Fv/Fm*, *NPQ*, *qI*) (Table 2). We observed that in both LL and HL conditions RILs with circadian rhythms closer to 24 hours had higher values of F_v'/F_m' (Fig. 2b, c), consistent with the experiment utilizing all 32 lines. The fold difference in *NPQ* under LL *vs*. HL conditions was associated with circadian period length (Fig. 4a), such that RILs with circadian periods longer than 24 hrs expressed fold differences closer to 1. Fold differences near 1 reflect RILs with comparatively high *NPQ* values even under the LL treatment suggesting those genotypes experienced surplus light energy that elicited a quenching requirement even in low light, a result akin to that observed in the clock mutants. We also observed an association between the fold difference in *qT* and circadian period (Fig. 4b). Specifically, RILs with shorter period lengths closer to 24 hr showed a ~1.5-fold increase in state-transition related quenching, *qT*, across the LL relative to HL environment, whereas the plants with period lengths closer to 27 hr had lower values across the two light treatments. Together, these findings suggest that there may be coordinated circadian regulation of photochemical (F_v'/F_m') and non-photochemical (*NPQ*) processes under two different levels of irradiance.

Principal Component Analysis

The PCA of data collected in the LL treatment revealed three major components that

317 describe genotypic variation (Table S1, Fig. S1a) and allow inference as to how different
318 traits (circadian period, A, g_s, chlorophyll fluorescence etc) are inter-related while
319 accounting for collinearity among multiple fluorescence measures. The first principal
320 component captured 43.95% of the total variance and was negatively related to *Fo*
321 (loading = -0.39), *Fm* (loading = -0.40), *Fo'* (loading = -0.41), and *Fm'* (loading = -0.40),
322 reflecting the well-known mathematical connection among fluorescence parameters. The
323 second principal component captured 28.47% of the variation and was positively related
324 to total *NPQ* (loading = 0.34), and negatively related to photosynthetic rates (loading = -
325 0.44), stomatal conductance (loading = -0.43), and *Fv'/Fm'* (loading = -0.42). The third
326 axis captured 10.77% of the variation and was positively related to circadian period
327 (loading = 0.52). Thus, PC2 and PC3 together account for variation that is independent of
328 fluorescence parameters *Fo, Fm, Fo', Fm'*. The loading of circadian period (PCA2) was
329 opposite in sign to that with *Fv'/Fm'* (PCA3) (Fig. S1a), consistent with the observed
330 negative bivariate correlation between these two traits (Fig. 2a, Table S1). PCA of gas
331 exchange and fluorescence traits in the HL treatment had similar trait loadings but were
332 generally less structured (inter-correlated), and specifically the association of the clock
333 and fold difference in *qT* (Fig. S1b) was absent, an outcome that could reflect light stress.
334 For HL, PC1 explained 43% of the total variance and was positively related to
335 fluorescence parameters *Fo, Fm, Fo', Fm'*. The second axes captured 20% and was
336 negatively related to parameters of gas-exchange (A, g_s) and *Fv'/Fm'* and positively
337 related to *NPQ*. The third and fourth axes both captured 11% of the variation were
338 positively related to *Fv/Fm* and circadian period. Overall, the PCA patterning is
339 consistent with univariate responses to the light treatments and observed bivariate

340 associations.

341 *Structural equation model*

342 To test the hierarchical relationships among measured circadian and physiological traits,
343 we used structural equation modeling. Based on AIC indices for all paths, we obtained a
344 model with good fit based on multiple metrics of Confirmatory Factor Analysis (Chi-
345 square p-value = 0.364, RMSEA = 0.026 ± 0.000 0.177 for the 90% CI, p-value = 0.466,
346 CFI = 0.999). The 'best fit' model is shown in Fig. 5a, and the standardized coefficients
347 for each of the modeled relationships are presented in Fig. 5b. The chi-square value of the
348 'best fit' model has a p-value > 0.05, which indicates that observed and expected
349 covariance matrices are not different and that the model has an adequate fit. The 90%
350 confidence interval (0.000-0.177) of the RMSEA indicates that the model has close
351 approximate fit to the data.

352 The SEM model revealed a network of connections between traits in the LL
353 treatment. As expected, photosynthetic rate (*A*) was regulated by stomatal conductance
354 (g_s) and *Fv'/Fm'*. Shorter circadian period (closer to 24 hrs) was associated with higher
355 *Fv'/Fm'* and lower values of *NPQ* (total non-photochemical quenching). *NPQ* was also
356 associated with stomatal conductance and *qE*. *qE* was the primary determinant of total
357 *NPQ*. The other two *NPQ* components, *qT* and *qI*, were removed during initial model
358 selection because they did not explain a significant proportion of the variance. Variation
359 in *qE* was also related to *A* and to *NPQ*. As expected from the traits' shared calculation
360 from fluorescence parameters, the decrease in *NPQ* was reflected in increased maximum
361 efficiency of PSII.

362

Discussion

Plants utilize the sun's energy as a source for photosynthesis. However, when plants experience light intensities that exceed the needs of photochemistry, excess excitation energy may be dissipated as heat or re-emitted as chlorophyll fluorescence. Excess radiation may impose significant stress and damage PSII (Björkman & Demmig-Adams, 1995; McDonald, 2003). Light availability and light stress vary in predictable ways over the course of the day such that quantitative clock variation is associated with gas-exchange in various species under field and controlled environmental conditions (Burstin *et al*., 2007; de Dios *et al*., 2016; Edwards *et al*., 2012; Edwards *et al*., 2011; Yarkhunova *et al*., 2016), and suggesting the circadian clock might contribute to regulation of thermal dissipation of excess energy. Here, we first quantified chlorophyll fluorescence patterns in mutant genotypes with disrupted clock function *vs*. genotypes with wild-type clock function. Using a segregating population, we then estimated the quantitative-genetic architecture of these traits, including estimation of genetic variances in gas-exchange traits, *NPQ*, and components of *NPQ* as well as of genetic correlations between these physiological traits and the circadian clock. We found significant connections between clock period and both PSII efficiency and non-photochemical quenching.

Wild-type clock function is associated with physiological parameters

Circadian regulation of physiological traits has been documented in a large number of studies and species (Dodd *et al*., 2014; Faure *et al*., 2012; Graf *et al*., 2010; McClung, 2013), and delayed fluorescence expresses circadian oscillations and is a proposed proxy

386 for circadian rhythms (Gould *et al*., 2009). Nevertheless, circadian regulation of the light
387 reactions of photosynthesis is not yet well-understood (Dodd *et al*., 2014). We were
388 interested in ascertaining whether clock function is related to *Fv'/Fm'* and to *NPQ* and its
389 components. Our results show that disruption of clock function via large-effect mutation
390 leads to shifts in *Fv'/Fm'* and *NPQ*, such that wild-type plants have both higher *Fv'/Fm'*
391 and lower total *NPQ*, representing more efficient photosynthetic machinery.
392
393 *Quantitative (co)variation of physiological traits and clock period*
394
395 Chlorophyll *a* fluorescence is frequently utilized to investigate PSII function and to
396 estimate the response of photosynthetic machinery to environmental stress (Baker &
397 Bowyer, 1994; Baker & Rosenqvist, 2004; Maxwell & Johnson, 2000). The energy-
398 dependent non-photochemical quenching component, *qE*, was the greatest contributor to
399 total *NPQ* under both high and low light, consistent with its role in protecting against
400 short-term high light and light fluctuations such as those that occurred between the
401 growth (350 μmol photons $m^{-2}s^{-1}$) and the measurement (500 μmol photons $m^{-2}s^{-1}$ or 1500
402 μmol photons $m^{-2}s^{-1}$) conditions (Demmig-Adams *et al*., 2014; Papageorgiou, 2014). On
403 average, the proportion of the *qE* component was higher among plants in the low light
404 treatment compared to high light treatment (although RILs also differed in the response
405 of this component to light treatment). The *qI* component of *NPQ* represents photodamage
406 to reaction centers of PSII (Demmig-Adams *et al*., 2014; Krause, 1988); on average over
407 all genotypes, *qI* values were correspondingly greater in the HL conditions. The role of
408 the *qT* component may lie in maximizing photosynthetic efficiency under low light

409 conditions, and the percentage of *qT* may therefore increase when light is limited
410 (Coopman *et al*., 2010, D'Ambrosio *et al*., 2008), which is consistent with our
411 observation of higher values of *qT* under low light conditions (Fig. 3c).
412 While many studies have characterized the genetic architecture of *A* (Edwards *et*
413 *al*., 2011, Fracheboud *et al*., 2002, Hervé *et al*., 2001, Teng *et al*., 2004) , fewer have
414 estimated genetic variances for *NPQ* and its component parameters (Jung & Niyogi,
415 2009, van Rooijen *et al*., 2015). We find significant genetic variances for *Fm'*, *Fv'/Fm'*,
416 *NPQ* and its individual components *qE*, *qT*, *qI*. Values of *Fv'/Fm'* ranged from 0.56 to
417 0.68 (Fig. 2a) among RILs, and *NPQ* values ranged from 1.1 to 1.8 in LL treatment. The
418 magnitude of *NPQ* variation among RILs is comparable to the magnitude of variation
419 observed among four accessions of *A. thaliana* (*NPQ* values = 1.5 to 2.0 at 600 μmol
420 photons $m^{-2}s^{-1}$) reported by Jung and Nigoyi (2009). We further observe variation among
421 RILs in *qE* (significant main effect of genotype on average across both treatments),
422 consistent with one prior study estimating genetic variances for *qE* among natural
423 accessions of *A. thaliana* (Niyogi *et al*., 2005). Interestingly, these phenotypic differences
424 observed among a small sample of RILs (or accessions in Jung and Nigoyi, 2009 and
425 Niyogi *et al*. 2005) are comparable to interspecific differences for *Fv'/Fm'* and *NPQ*
426 (Demmig-Adams *et al*., 2006; Guo & Trotter, 2004), indicating that segregating variation
427 in a within-species cross can reproduce phenotypic differences among species
428 Previous studies have found that circadian periods providing a match to
429 environmental conditions are beneficial for plant growth and performance under
430 controlled conditions (Barak *et al*., 2000; Yerushalmi & Green, 2009) and in the field
431 (Rubin *et al*., 2017), and can lead to higher gas-exchange values (Dodd et al. 2005;

Edwards et al. 2011; Yarkhunova et al. 2016). Further, many genes encoding proteins associated with PSII functioning and *NPQ* (PsbS protein and other Psb subunits) are circadian regulated (Covington *et al*., 2008), suggesting the clock may regulate PSII efficiency. We observe that circadian period lengths among a set of *A. thaliana* RILs varies from 24 to 27 hours, and that this quantitative variation in circadian period correlates with chlorophyll *a* fluorescence parameters. In addition, our data indicate that this relationship is maintained under three different light conditions (Fig. 2a, b, c). This association in the RILs together with the clock mutant results suggest that the adaptive value of the circadian clock may arise in part from regulation of PSII function (Kreps & Simon, 1997).

In addition to *Fv'/Fm'*, we observe that plants with high fold changes in *NPQ* across low- to high-light conditions have period lengths that deviate from (are longer than) 24 hrs. Genotypes with a circadian period closer to 27 hrs have higher initial rates of *NPQ* under low light, indicating that the photoprotective mechanisms are induced at lower light levels compared to the lines with shorter period lengths. These observations demonstrate that there is a change in PSII excitation balance (Huner *et al*., 1998) among long-period genotypes such that even LL imposes stress, providing a further indication that the clock is linked to PS II. We observed that genotypes with a circadian period closer to 24 hr show comparatively greater values of *qT* under LL (500 μmol photons $m^{-2}s^{-1}$) *vs*. HL (1500 μmol photons $m^{-2}s^{-1}$) conditions (Fig. 4b), a pattern that is consistent with the view that at least wild-type *A. thaliana* are generally not stressed at low light levels of 500 μmol photons $m^{-2}s^{-1}$ and may in fact be light limited (Bailey *et al*., 2004). Plants with normal clock function (expressing 24 hr periods) may experience light

455 limitation at 500 μmol photons $m^{-2}s^{-1}$. Transcriptomic studies reveal that some genes that
456 code for enzymes that are required for state transitions (STN7 protein kinase,
457 AT1G68830, AT5G01920, AT4G27800) are circadian regulated (Covington *et al*., 2008),
458 suggesting the clock plays an important role in synchronization of state transitions. It is
459 worth noting that neither *qE* nor *qI* showed correlations with circadian period in our
460 study, and neither the genes responsible for *qE* sites such as LHCII, CP29, and CP26
461 (AT1G19150, AT3G53460, AT4G10340), nor the genes associated with photoinhibition
462 (AT1G77510, AT2G30950, AT3G19570) are under circadian control (Covington *et al*.,
463 2008).
464
465 *PCA and Path analysis confirmed empirical relationships between physiological traits*
466
467 Three groups of traits that contribute to variation among the genotypes were identified
468 using the PCA analysis. The first group includes the fluorescence parameters *Fo*, *Fm*,
469 *Fo'*, and *Fm'*. All of these parameters are related and reflect physical properties of the
470 primary quinone acceptor of PSII, Q_A, or are partly influenced by PSII reaction center
471 redox activities (Roháček, 2002). The second group of traits contributes to variation in
472 *NPQ*, *Fv'*/*Fm'*, and gas-exchange traits; the third one is related to circadian period. PCA
473 and structural equation modeling revealed the correlation structure of complex traits and
474 potential mechanistic relationships, including how circadian period both directly and
475 indirectly interacts with and might influence physiological trait expression (Fig. 5; Fig.
476 S1).

Most of the paths in the SEM model were supported by bivariate correlations and PC analysis, and specifically supported clock associations with chlorophyll fluorescence. As noted, thermal dissipation, chlorophyll fluorescence and photochemistry (primarily photosynthesis) are the three possible fates of light energy in the leaf, and all three occur simultaneously (Baker, 2008), and therefore associations among components of *NPQ* as well as between *A* and at least some chlorophyll fluorescence measures are anticipated. Our SEM results are consistent with other studies, showing that *qE* is the primary contributor to *NPQ* (Niyogi *et al*., 2005). Further, *NPQ* does not directly affect *A*, but instead acts indirectly through *Fv'/Fm'*. This indirect relationship likely reflects the fact that *NPQ* (in contrast to PSII activity) does not result in ATP or NADPH production for the Calvin Benson cycle, but instead dissipates excitation energy as heat (Ruban *et al*., 2016). Although we do not observe a significant path between total *NPQ* and *A*, our results show that the *qE* component of *NPQ* negatively affects *A*. *qE* regulates the excitation rate of PSII reaction centers, which might contribute to energy utilization in the photosynthetic apparatus and thereby affect values of *A* through the production of ATP and NADPH. The SEM also reveal an association between circadian period and both *Fv'/Fm'* and *NPQ*. In sum, our results from clock mutants and segregating lines are consistent with the hypothesized importance of a functional circadian clock that resonates with ambient conditions to plant growth, survival and reproduction (Dodd *et al*., 2005, Edwards *et al*., 2011, Green *et al*., 2002, Salmela *et al*., 2015, Yarkhunova *et al*., 2016).

Conclusions

The circadian clock has been implicated in plant performance in controlled settings, in which alleles conferring a match between endogenous rhythms and diurnal cycles evolve to higher frequency (Yerushalmi & Green, 2009) as well as in field settings, in which discrete and quantitative clock phenotypes are associated with differences in allocation (Salmela *et al*., 2015) and in survival and fruit set (Rubin *et al*. 2017). The underlying physiological reasons for these performance differences are unknown, although quantitative clock variation correlates with gas-exchange traits (Edwards *et al*., 2012, Yarkhunova *et al*., 2016). Recent studies also indicate that natural variation at the clock gene, *GIGANTEA*, affects cold tolerance (Xie *et al*., 2015) and growth patterns (de Montaigu *et al*., 2015) while in domesticated tomato delayed circadian clock was selected during the process of domestication (Müller *et al*., 2016). Our data suggest that circadian rhythms might play an important role in regulation of plant photosynthetic machinery. Specifically, the results of the present study suggest possible circadian regulation of maximum efficiency of PSII, *NPQ* and the *qT* component of *NPQ*.

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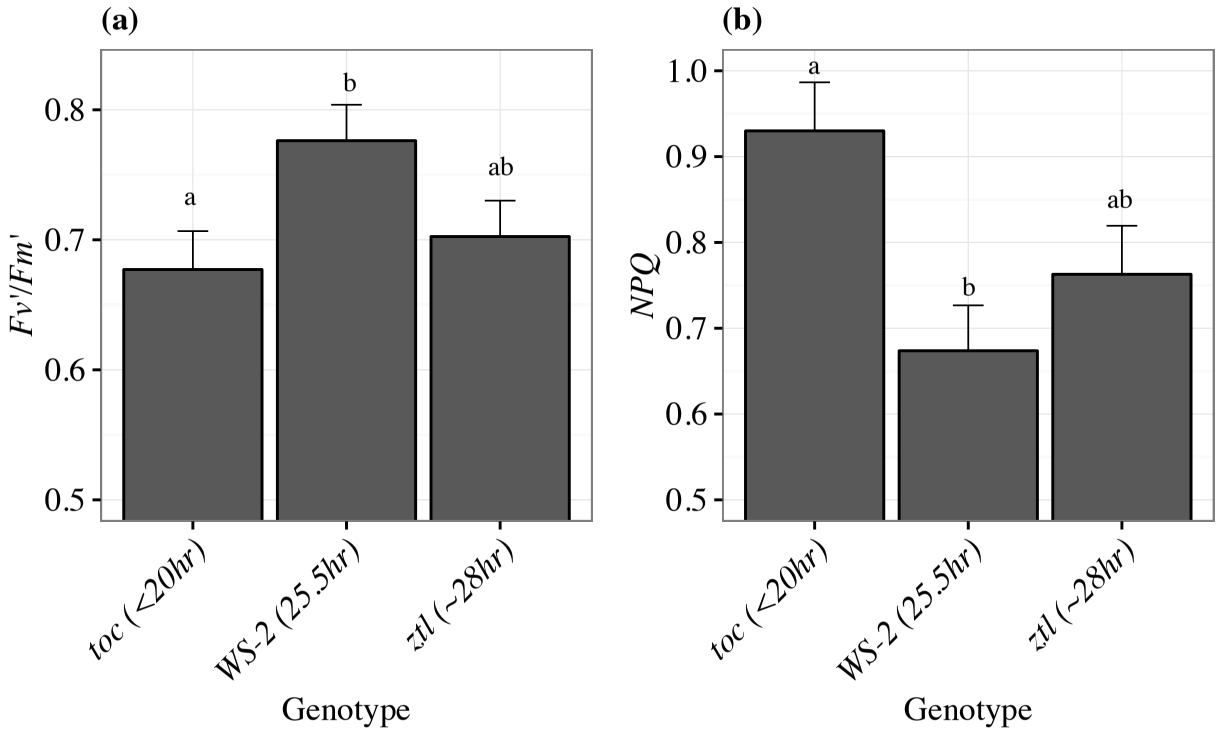

(a)
Fv'/Fm'
0.8
0.7
0.6
0.5
a
b
ab
toc (<20hr)
WS-2 (25.5hr)
ztl (~28hr)
Genotype
(b)
NPQ
1.0
0.9
0.8
0.7
0.6
0.5
a
b
ab
toc (<20hr)
WS-2 (25.5hr)
ztl (~28hr)
Genotype

Figure 1. Differences in quantum yield of PSII (*Fv'/Fm'*) **(a)** and total non-photochemical quenching *NPQ* **(b)** among circadian clock mutant and wild type genotypes of *Arabidopsis thaliana* growing at 350 μmol photons m^{-2} s^{-1}, 22 ± 1°C. Error bars indicate ± SE. Different letters indicate statistically significant differences among ($p < 0.05$).

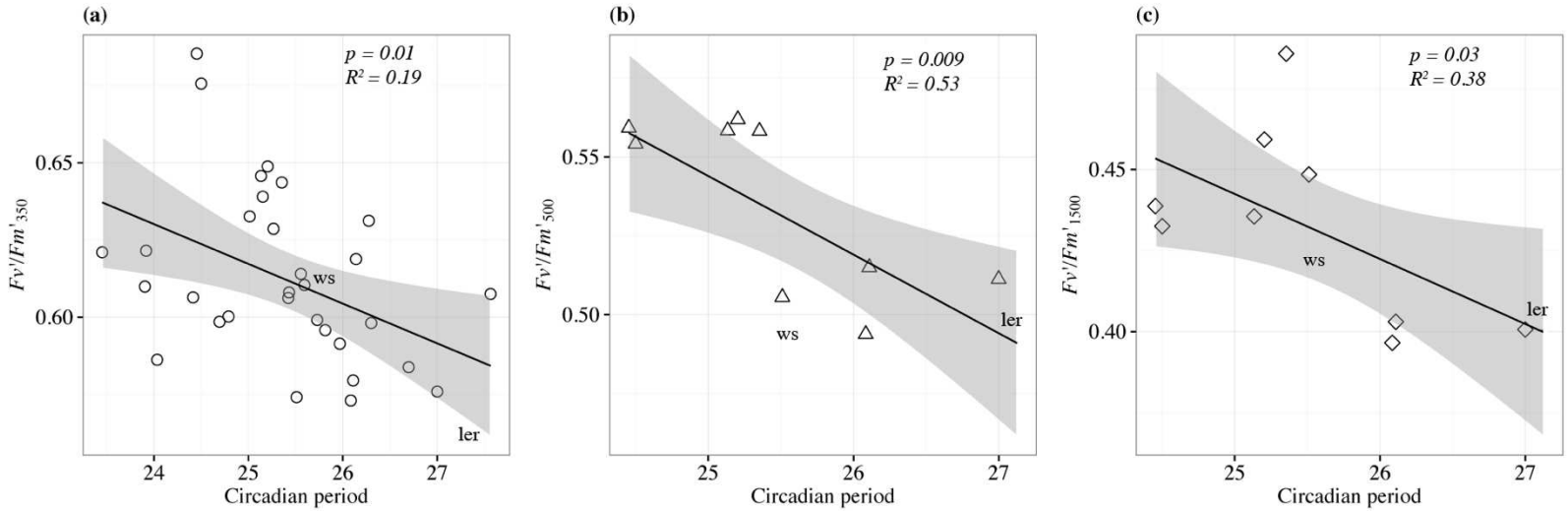

(a)
p = 0.01
R² = 0.19
0.65
0.60
Fv'/Fm'350
ws
ler
24
25
26
27
Circadian period
(b)
p = 0.009
R² = 0.53
0.55
0.50
Fv'/Fm'500
ws
ler
25
26
27
Circadian period
(c)
p = 0.03
R² = 0.38
0.45
0.40
Fv'/Fm'1500
ws
ler
25
26
27
Circadian period

Figure 2. Association between circadian period and quantum yield of photosystem II (Fv'/Fm') at different light levels.

(a) Association between circadian period and F_v'/F_m' for thirty-two *Arabidopsis thaliana* genotypes at 350 μmol photons m^{-2} s^{-1}. Each circle represents a genotype while *ws* and *ler* represents the parental genotypes. The line represents the following relationship: R^2=0.19, *p*=0.01

(b) Association between circadian period and F_v'/F_m' in low light condition (500 μmol photons m^{-2} s^{-1}; LL) for eleven *Arabidopsis thaliana* genotypes. Each triangle represents a genotype. The line represents the following relationship: R^2=0.53, *p*=0.0099

(c) Association between circadian period and F_v'/F_m' in high light conditions (1500 μmol photons m^{-2} s^{-1}; HL) for eleven *Arabidopsis thaliana* genotypes. Each diamond represents a genotype. The line represents the following relationship: R^2=0.38, *p*=0.03

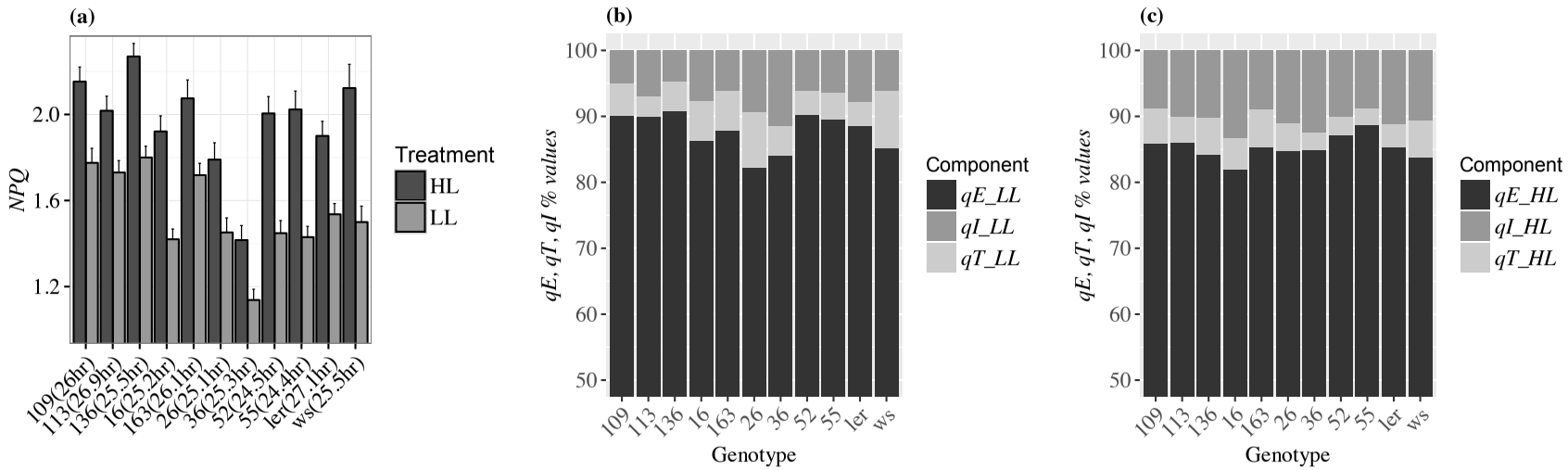

(a)
NPQ
2.0
1.6
1.2
109(26hr)
113(26.9hr)
136(25.5hr)
16(25.2hr)
163(26.1hr)
26(25.1hr)
36(25.3hr)
52(24.5hr)
55(24.4hr)
ler(27.1hr)
ws(25.5hr)
Treatment
HL
LL
(b)
qE, qT, qI % values
100
90
80
70
60
50
109
113
136
16
163
26
36
52
55
ler
ws
Genotype
Component
qE_LL
qI_LL
qT_LL
(c)
qE, qT, qI % values
100
90
80
70
60
50
109
113
136
16
163
26
36
52
55
ler
ws
Genotype
Component
qE_HL
qI_HL
qT_HL

Figure 3. (a) Differences in total *NPQ* among RILs of *Arabidopsis thaliana* under different light conditions (500 µmol photons m^{-2} $s^{-1,}$ LL and 1500 µmol photons m^{-2} s^{-1}, HL). (**b)** Individual *NPQ* components (*qE*, *qT* and *qI*) expressed as percentage values in leaves of *A. thaliana* RIL genotypes measured at 500 µmol photons m^{-2} $s^{-1,}$ LL and (**c**) at 1500 µmol photons m^{-2} s^{-1}, HL

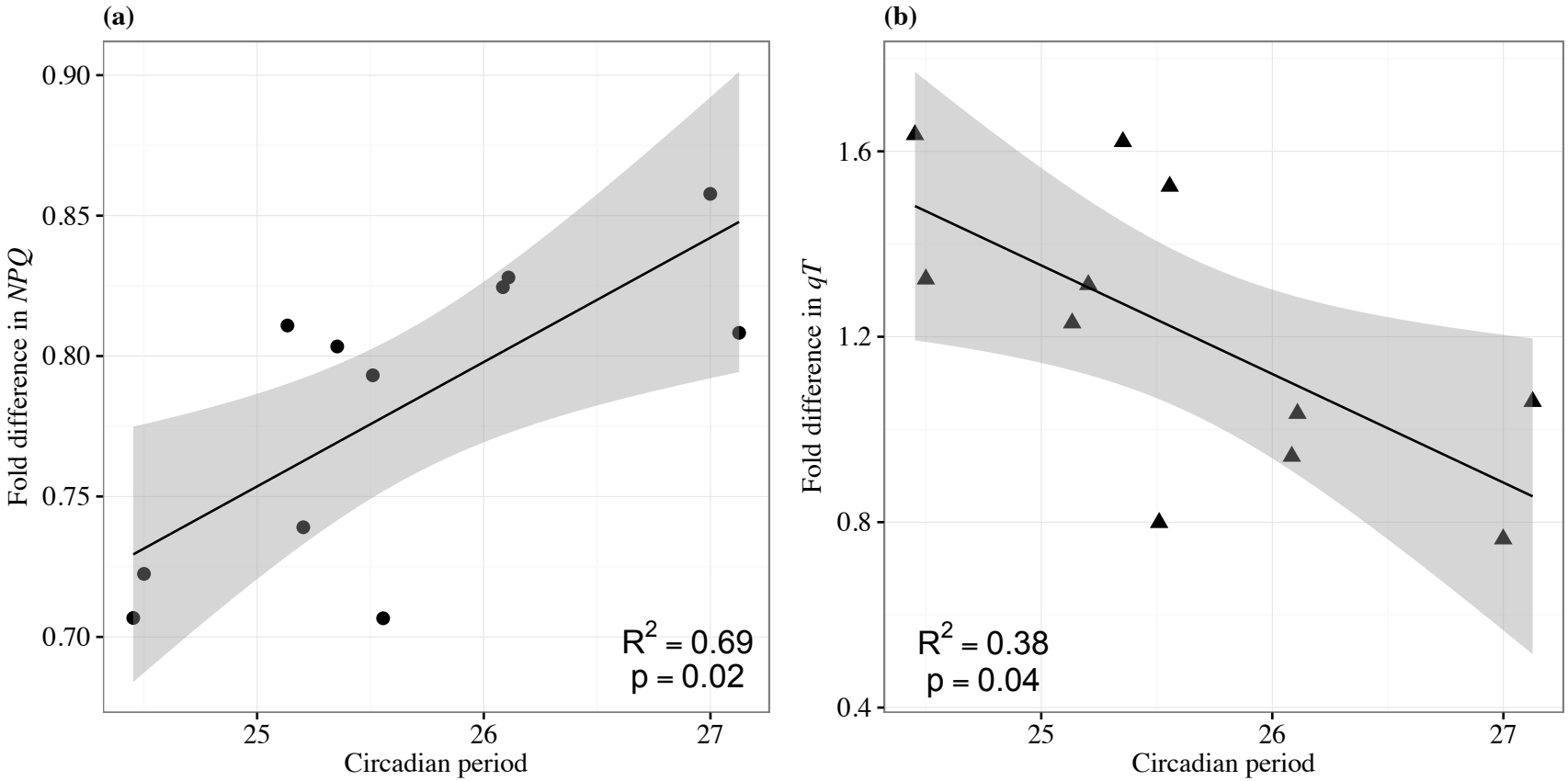
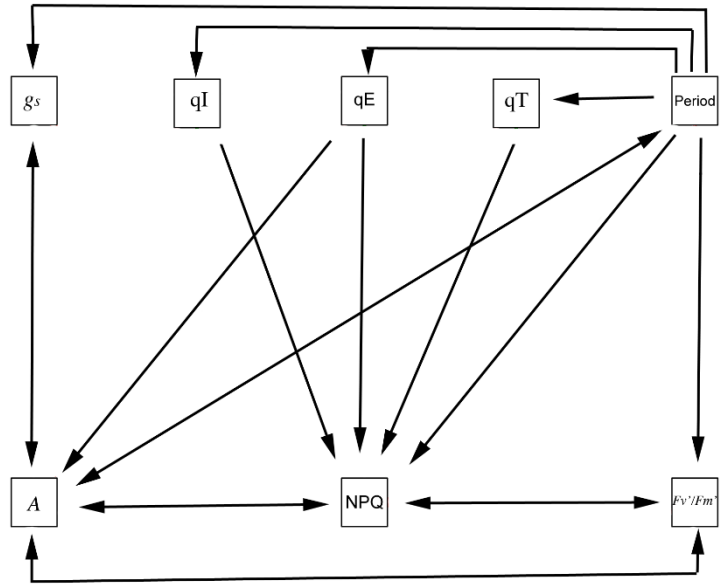

(a)
Fold difference in NPQ
0.90
0.85
0.80
0.75
0.70
25
26
27
Circadian period
R2 = 0.69
p = 0.02
(b)
Fold difference in qT
1.6
1.2
0.8
0.4
25
26
27
Circadian period
R2 = 0.38
p = 0.04

Figure 4. **Association between circadian period and fold difference of *NPQ*.**

(a) Association between circadian period and fold difference of total *NPQ* (values under LL / HL) for eleven *Arabidopsis thaliana* RIL genotypes. Each circle represents a genotype while *ws* and *ler* represents the parental genotypes. The line represents the following relationship: R^2=0.44, *p*=0.02

(b) Association between circadian period and transitionary quenching (*qT*) for eleven *Arabidopsis thaliana* genotypes. Each triangle represents a genotype while *ws* and *ler* represents the parental genotypes. The line represents the following relationship: R^2=0.38, *p*=0.04

(a)


gs
qI
qE
qT
Period
A
NPQ
Fv'/Fm'

(b)

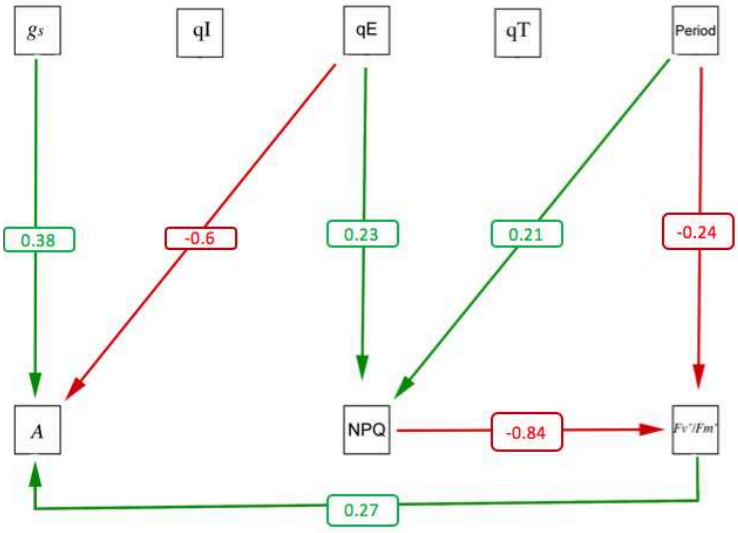

gs
qI
qE
qT
Period
0.38
-0.6
0.23
0.21
-0.24
A
NPQ
-0.84
Fv'/Fm'
0.27

Figure 5. **(a)** Tested model **(b)** Path diagram of the relationships among physiological traits and circadian period of *A. thaliana* at 500 μmol photons m^{-2} s^{-1}, (LL) light treatment. *Arrows* indicate significant relationships. Labels on arrows show standardized path coefficients. Paths are drawn with solid green lines if positive and red lines if negative, $n = 95$.

Table 1a. Analysis of variance for effects of circadian clock genotype on *Fv'/Fm'* and *NPQ*.

Fv'/Fm'

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	2	0.04061530	0.02030765	3.32	0.0568

NPQ

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	2	0.24957873	0.12478937	5.57	0.0125

Table 1b. Analysis of variance for effects of RIL genotype on circadian period.

Circadian Period					
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Genotype	31	215.0204579	6.9361438	11.58	<.0001

Table 1c. Analysis of variance for effects of genotype and treatment (LL and HL) on gas-exchange parameters and components of photochemical and non-photochemical quenching.

Fm'

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	1790663.954	179066.395	9.15	<.0001
Treatment	1	2946925.899	2946925.899	150.6	<.0001
Genotype*Treatment	10	451899.95	45189.995	2.31	0.0148

A

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	356.0066621	35.6006662	3.46	0.0004
Treatment	1	39.2847737	39.2847737	3.82	0.0522
Genotype*Treatment	10	68.6918485	6.8691849	0.67	0.7527

g_s

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	0.20292674	0.02029267	5.78	<.0001
Treatment	1	0.00073165	0.00073165	0.21	0.6485
Genotype*Treatment	10	0.03881545	0.00388155	1.11	0.3603

Fv'/Fm'

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	0.15260004	0.01526	18.3	<.0001
Treatment	1	0.38823759	0.38823759	465.49	<.0001
Genotype*Treatment	10	0.01960153	0.00196015	2.35	0.0132

NPQ

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	8.27940223	0.82794022	26.43	<.0001
Treatment	1	7.27058433	7.27058433	232.06	<.0001
Genotype*Treatment	10	0.5065335	0.05065335	1.62	0.1068

qE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	0.05566935	0.00556694	4.86	<.0001
Treatment	1	0.02865255	0.02865255	25.01	<.0001
Genotype*Treatment	10	0.02556133	0.00255613	2.23	0.0189

qI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	0.0390058	0.00390058	5.48	<.0001
Treatment	1	0.04846508	0.04846508	68.1	<.0001
Genotype*Treatment	10	0.0091006	0.00091006	1.28	0.2475

qT

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	10	0.02075992	0.00207599	6.43	<.0001
Treatment	1	0.0012175	0.0012175	3.77	0.054
Genotype*Treatment	10	0.00522974	0.00052297	1.62	0.106

Table 2. Phenotypic correlations between traits in *Arabidopsis* RIL population in LL light treatment. * P<0.05; ** P<0.01; *** P<0.001; ns not significant

	Period	***A***	G_s	F_v'/F_m'	F_o	F_v/F_m	**NPQ**	F_m	**qE**	**qT**	**qI**
Period	1	-0.18424^{ns}	-0.3255^{ns}	-0.73518**	-0.34427^{ns}	-0.01184^{ns}	0.4895^{ns}	-0.34318^{ns}	0.18781^{ns}	-0.25292^{ns}	-0.03305^{ns}
A	-0.18424^{ns}	1	0.91449***	0.66855**	-0.05218^{ns}	0.78373**	-0.77557**	0.24886^{ns}	-0.42351^{ns}	-0.13583^{ns}	0.74798**
G_s	-0.3255^{ns}	0.91449***	1	0.65384*	0.02668^{ns}	0.64053*	-0.89794**	0.2743^{ns}	-0.43889^{ns}	-0.1152^{ns}	0.75076**
F_v'/F_m'	-0.73518**	0.66855*	0.65384*	1	0.09073^{ns}	0.33412^{ns}	-0.71858**	0.21581^{ns}	-0.39723^{ns}	0.04947^{ns}	0.53288^{ns}
F_o	-0.34427^{ns}	-0.05218^{ns}	0.02668^{ns}	0.09073^{ns}	1	-0.01753^{ns}	-0.19731^{ns}	0.92043***	-0.32823^{ns}	0.37615^{ns}	0.12059^{ns}
F_v/F_m	-0.01184^{ns}	0.78373**	0.64053*	0.33412^{ns}	-0.01753^{ns}	1	-0.50506^{ns}	0.37281^{ns}	-0.45395^{ns}	0.20493^{ns}	0.46745^{ns}
NPQ	0.4895^{ns}	-0.77557**	-0.89794**	-0.71858**	-0.19731^{ns}	-0.50506^{ns}	1	-0.38731^{ns}	0.6344*	-0.13819^{ns}	-0.7946**
F_m	-0.34318^{ns}	0.24886^{ns}	0.2743^{ns}	0.21581^{ns}	0.92043***	0.37281^{ns}	-0.38731^{ns}	1	-0.48925^{ns}	0.44896^{ns}	0.28628^{ns}
qE	0.18781^{ns}	-0.42351^{ns}	-0.43889^{ns}	-0.39723^{ns}	-0.32823^{ns}	-0.45395^{ns}	0.6344*	-0.48925^{ns}	1	-0.7331**	-0.76113**
qT	-0.25292^{ns}	-0.13583^{ns}	-0.1152^{ns}	0.04947^{ns}	0.37615^{ns}	0.20493^{ns}	-0.13819^{ns}	0.44896^{ns}	-0.7331**	1	0.11686^{ns}
qI	-0.03305^{ns}	0.74798**	0.75076**	0.53288^{ns}	0.12059^{ns}	0.46745^{ns}	-0.7946**	0.28628^{ns}	-0.76113**	0.11686^{ns}	1