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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 CO₂, 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 ($F_v'/F_m'$) 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 $F_v'/F_m'$, 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 $F_v'/F_m'$ 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 $F_v'/F_m'$ 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 &
Demmig-Adams, 1995; McDonald, 2003). Further, the efficiency of photosystem II (PSII) in utilizing light energy (Fv’/Fm’) correlates with gas-exchange rates and plant performance under various experimental conditions at a given light level (Maxwell & Johnson, 2000). However, the absorbed light energy may exceed the demand for energy and the reducing capacity of the light-independent reactions of photosynthesis, potentially leading to photodamage through formation of reactive oxygen species (ROS).

In response to light stress, plants have evolved several photoprotective mechanisms. A large number of enzymes take part in scavenging activities (Asada, 2006; Das & Roychoudhury, 2014); some carotenoids have been shown to be highly efficient in scrubbing excited chlorophyll molecules (Bassi & Caffarri, 2000), and ascorbate is also an efficient antioxidant in various organisms (Fukumura et al., 2012). However, when excitation energy exceeds demand, the first line of defense to avoid damage to PSII is heat dissipation. Thermal dissipation is a protective strategy to reduce photoinhibition, and is ubiquitous to photosynthetic organisms (Müller et al., 2001). This mechanism competes with photochemistry and chlorophyll a fluorescence for the use of excitation energy (Baker, 2008), and it is commonly referred to as non-photochemical quenching of chlorophyll a fluorescence (NPQ).

NPQ comprises at least three major components: qE (pH-dependent quenching), qT (state-transition quenching) and qI (quenching related to photoinhibition). The onset of qE occurs quickly, within seconds to a few minutes, and is triggered through the synergistic action of thylakoid lumen pH and the formation of an energy quenching complex between the protein PsbS and the pool of xanthophyll and zeaxanthin (Horton et al., 2000; Li et al., 2002). The qT component can occur following 2-15 minutes of
illumination and reflects the balance of excitation between the two photosystems, which depends upon reversible photophosphorylation activity and ensuing relocation of light harvesting complexes (Niyogi, 2000). $q_I$ has slow relaxation kinetics and is related directly to photoinhibition, including down-regulation and complete deactivation of PSII (Li et al., 2002).

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

Light availability and light stress vary in predictable ways over the course of the day. Quantitative clock variation is correspondingly 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 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
short clock period (20 hr), and the cognate, Ws-2, wild-type genotype in which these mutations reside.

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

To characterize genetic and environmental sources of variation in Fv'/Fm', components of NPQ, and associations between these two traits and clock period, we used recombinant inbred lines (RILs) of Arabidopsis thaliana (L.) Heynh. (Brassicaceae). The RILs were developed from a cross between Ler (Landsberg erecta, Germany) and Ws-2 (Wassilewskaja, Belarus), in which the Ws-2 parent harbors the reporter gene LUCIFERASE (LUC) linked to the promoter of COLD-CIRCADIAN RHYTHM–RNA BINDING 2 (CCR2), allowing for quantification of circadian parameters (Millar, Short, Chua & Kay, 1992). Details of the crossing design are provided in Boikoglou & Davis (2009) and Rubin et al (2017). In brief, the two parents were crossed to create a
heterozygous F₁. The F₁ was then backcrossed to the maternal parent, and the resulting
BC₁F₂ genotypes were selfed to the BC₁F₆ 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) $\mu$mol m$^{-2}$ s$^{-1}$, flow rate= 300 m$^{-2}$ s$^{-1}$,
- $\text{ref } [\text{CO}_2] = 400 \mu$mol m$^{-2}$ s$^{-1}$, $T_{\text{leaf}}= 22^\circ\text{C}$ 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 $\mu$mol 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 $F_o'$ used the equation from Oxborough and Baker (1997), $F_o'=F_o/(F_v/F_m'+F_o/F_m')$. 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 $\mu$mol 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
decays. The half-times for each component were reported as qI = A, qT = (B − A), qE = (C − B), with A, B and C intercepts on the y axis (D’Ambrosio et al., 2008).

Data analysis

Statistical approach and data treatments

All analyses were conducted in R version 3.2.4 (Team, 2014), http://www.r-project.org. Analysis of variance (ANOVA) was used to test for differences in Fv'/Fm' and total NPQ between wild-type and clock mutant genotypes in the first experiment. ANOVA was also used to test the influence of light treatments and genotypic effect on physiological traits (including circadian period, Fv'/Fm', total NPQ, A, g_s, qE, qT, qI) measured in the RILs (‘lm’ and ‘anova’ functions of R). Further, we estimated the fold difference in NPQ or its components by dividing the trait value in one light treatment by its value in the other treatment (low light / high light treatment). Principal components analysis (PCA) was performed using the ‘prcomp’ procedure in R, and scores were tested for the effect of genotype.

We were further interested in testing the relative contribution of individual physiological traits and circadian period to the expression of A_max. First, we determined how clusters of traits related to genetic variation in the RILs using Principal Components Analysis (PCA) as an approach to address collinearity between fluorescence variables. Second, to quantify hypothesized causal relationships between traits, we used structural equation modeling with observed variables. We developed an initial (saturated) model
based on observed bivariate correlations and known relationships among physiological
traits and between circadian and physiological traits. The fit of alternative structural
equation models to the observed data was tested with the sem() function of the ‘lavaan’
package (Rosseel, 2012) in R version 3.2.4 (Team, 2014). To identify a model with good
fit, a proposed model was evaluated through Confirmatory Factor Analysis within the
lavaan package and the fit indices that rank parsimony (Akaike’s Information Criterion;
AIC). If the fit criteria (described below) were not met for the proposed model, then
modification indices were used to adjust the model; specifically, variables were excluded
from the model with the highest AIC, and fit indices for the reduced model were again
evaluated. Model fit was assessed with a chi-square test, root mean square error of
approximation (RMSEA), and comparative fit index (CFI). Chi-square values associated
with a P-value > 0.05 and a RMSEA <0.05 and CFI > 0.95 indicate a good fit of the
model to the data (Kline, 2015).

Once the model with the best fit was identified, structural equation modeling was
used to partition variation in a response variable among multiple predictor variables.
Specifically, the multivariate regression model that is the basis for structural equation
modeling statistically accounts for variation in multiple predictor variables (in this case,
traits) simultaneously and tests their relationship to a response variable. We were
interested in the hierarchical relationships among measured traits (e.g., circadian period,
gas-exchange traits, NPQ). This approach reveals the extent to which a given trait
directly vs. indirectly affects the response variable (e.g., circadian period could affect $A_{\text{max}}$
directly or act indirectly through NPQ) (e.g., Fournier-Level et al, 2013).
Results

To test for a clock effect on chlorophyll fluorescence, we compared $F_{v'}/F_{m'}$ 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 ($F_{v'}/F_{m'}$) (Table 1a). Specifically, wild type Ws-2 plants had higher values of $F_{v'}/F_{m'}$ 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 ($F_{v'}/F_{m'}$) 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 $F_{v'}/F_{m'}$ 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 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) and high light, HL, 1500 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) 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
describe genotypic variation (Table S1, Fig. S1a) and allow inference as to how different
traits (circadian period, A, gs, chlorophyll fluorescence etc) are inter-related while
accounting for collinearity among multiple fluorescence measures. The first principal
component captured 43.95% of the total variance and was negatively related to Fo
(loadings = 0.39), Fm (loading = 0.40), Fo' (loading = 0.41), and Fm' (loading = 0.40),
reflecting the well-known mathematical connection among fluorescence parameters. The
second principal component captured 28.47% of the variation and was positively related
to total NPQ (loading = 0.34), and negatively related to photosynthetic rates (loading = -
0.44), stomatal conductance (loading = -0.43), and Fv'/Fm' (loading = -0.42). The third
axis captured 10.77% of the variation and was positively related to circadian period
(loadings = 0.52). Thus, PC2 and PC3 together account for variation that is independent of
fluorescence parameters Fo, Fm, Fo', Fm'. The loading of circadian period (PCA2) was
opposite in sign to that with Fv'/Fm' (PCA3) (Fig. S1a), consistent with the observed
negative bivariate correlation between these two traits (Fig. 2a, Table S1). PCA of gas
exchange and fluorescence traits in the HL treatment had similar trait loadings but were
generally less structured (inter-correlated), and specifically the association of the clock
and fold difference in qT (Fig. S1b) was absent, an outcome that could reflect light stress.
For HL, PC1 explained 43% of the total variance and was positively related to
fluorescence parameters Fo, Fm, Fo', Fm'. The second axes captured 20% and was
negatively related to parameters of gas-exchange (A, gs) and Fv'/Fm' and positively
related to NPQ. The third and fourth axes both captured 11% of the variation were
positively related to Fv/Fm and circadian period. Overall, the PCA patterning is
consistent with univariate responses to the light treatments and observed bivariate
To test the hierarchical relationships among measured circadian and physiological traits, we used structural equation modeling. Based on AIC indices for all paths, we obtained a model with good fit based on multiple metrics of Confirmatory Factor Analysis (Chi-square p-value = 0.364, RMSEA = 0.026 ± 0.000 0.177 for the 90% CI, p-value = 0.466, CFI = 0.999). The ‘best fit’ model is shown in Fig. 5a, and the standardized coefficients for each of the modeled relationships are presented in Fig. 5b. The chi-square value of the ‘best fit’ model has a p-value > 0.05, which indicates that observed and expected covariance matrices are not different and that the model has an adequate fit. The 90% confidence interval (0.000-0.177) of the RMSEA indicates that the model has close approximate fit to the data.

The SEM model revealed a network of connections between traits in the LL treatment. As expected, photosynthetic rate (A) was regulated by stomatal conductance \( g_s \) and \( F_{v'/Fm'} \). Shorter circadian period (closer to 24 hrs) was associated with higher \( F_{v'/Fm'} \) and lower values of \( NPQ \) (total non-photochemical quenching). \( NPQ \) was also associated with stomatal conductance and \( qE \). \( qE \) was the primary determinant of total \( NPQ \). The other two \( NPQ \) components, \( qT \) and \( qI \), were removed during initial model selection because they did not explain a significant proportion of the variance. Variation in \( qE \) was also related to \( A \) and to \( NPQ \). As expected from the traits’ shared calculation from fluorescence parameters, the decrease in \( NPQ \) was reflected in increased maximum efficiency of PSII.
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
for circadian rhythms (Gould et al., 2009). Nevertheless, circadian regulation of the light reactions of photosynthesis is not yet well-understood (Dodd et al., 2014). We were interested in ascertaining whether clock function is related to $F_{v'}/F_{m'}$ and to NPQ and its components. Our results show that disruption of clock function via large-effect mutation leads to shifts in $F_{v'}/F_{m'}$ and NPQ, such that wild-type plants have both higher $F_{v'}/F_{m'}$ and lower total NPQ, representing more efficient photosynthetic machinery.

Quantitative (co)variation of physiological traits and clock period

Chlorophyll a fluorescence is frequently utilized to investigate PSII function and to estimate the response of photosynthetic machinery to environmental stress (Baker & Bowyer, 1994; Baker & Rosenqvist, 2004; Maxwell & Johnson, 2000). The energy-dependent non-photochemical quenching component, $qE$, was the greatest contributor to total NPQ under both high and low light, consistent with its role in protecting against short-term high light and light fluctuations such as those that occurred between the growth (350 μmol photons m$^{-2}$s$^{-1}$) and the measurement (500 μmol photons m$^{-2}$s$^{-1}$ or 1500 μmol photons m$^{-2}$s$^{-1}$) conditions (Demmig-Adams et al., 2014; Papageorgiou, 2014). On average, the proportion of the $qE$ component was higher among plants in the low light treatment compared to high light treatment (although RILs also differed in the response of this component to light treatment). The $qI$ component of NPQ represents photodamage to reaction centers of PSII (Demmig-Adams et al., 2014; Krause, 1988); on average over all genotypes, $qI$ values were correspondingly greater in the HL conditions. The role of the $qT$ component may lie in maximizing photosynthetic efficiency under low light.
conditions, and the percentage of $q_T$ may therefore increase when light is limited (Coopman et al., 2010, D’Ambrosio et al., 2008), which is consistent with our observation of higher values of $q_T$ under low light conditions (Fig. 3c).

While many studies have characterized the genetic architecture of A (Edwards et al., 2011, Fracheboud et al., 2002, Hervé et al., 2001, Teng et al., 2004), fewer have estimated genetic variances for $NPQ$ and its component parameters (Jung & Niyogi, 2009, van Rooijen et al., 2015). We find significant genetic variances for $Fm'$, $Fv'/Fm'$, $NPQ$ and its individual components $q_E$, $q_T$, $q_I$. Values of $Fv'/Fm'$ ranged from 0.56 to 0.68 (Fig. 2a) among RILs, and $NPQ$ values ranged from 1.1 to 1.8 in LL treatment. The magnitude of $NPQ$ variation among RILs is comparable to the magnitude of variation observed among four accessions of A. thaliana ($NPQ$ values = 1.5 to 2.0 at 600 μmol photons m$^{-2}$s$^{-1}$) reported by Jung and Nigoyi (2009). We further observe variation among RILs in $q_E$ (significant main effect of genotype on average across both treatments), consistent with one prior study estimating genetic variances for $q_E$ among natural accessions of A. thaliana (Niyogi et al., 2005). Interestingly, these phenotypic differences observed among a small sample of RILs (or accessions in Jung and Nigoyi, 2009 and Niyogi et al. 2005) are comparable to interspecific differences for $Fv'/Fm'$ and $NPQ$ (Demmig-Adams et al., 2006; Guo & Trotter, 2004), indicating that segregating variation in a within-species cross can reproduce phenotypic differences among species.

Previous studies have found that circadian periods providing a match to environmental conditions are beneficial for plant growth and performance under controlled conditions (Barak et al., 2000; Yerushalmi & Green, 2009) and in the field (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 \(Fv'\text{/}Fm'\), 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 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\)) vs. HL (1500 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\)) 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 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\) and may in fact be light limited (Bailey et al., 2004). Plants with normal clock function (expressing 24 hr periods) may experience light
limitation at 500 μmol photons m$^{-2}$s$^{-1}$. Transcriptomic studies reveal that some genes that
code for enzymes that are required for state transitions (STN7 protein kinase, AT1G68830, AT5G01920, AT4G27800) are circadian regulated (Covington et al., 2008), suggesting the clock plays an important role in synchronization of state transitions. It is worth noting that neither $qE$ nor $qI$ showed correlations with circadian period in our study, and neither the genes responsible for $qE$ sites such as LHCII, CP29, and CP26 (AT1G19150, AT3G53460, AT4G10340), nor the genes associated with photoinhibition (AT1G77510, AT2G30950, AT3G19570) are under circadian control (Covington et al., 2008).

*PCA and Path analysis confirmed empirical relationships between physiological traits*

Three groups of traits that contribute to variation among the genotypes were identified using the PCA analysis. The first group includes the fluorescence parameters $F_o$, $F_m$, $F_o'$, and $F_m'$. All of these parameters are related and reflect physical properties of the primary quinone acceptor of PSII, $Q_A$, or are partly influenced by PSII reaction center redox activities (Roháček, 2002). The second group of traits contributes to variation in $NPQ$, $F_{v'}/F_m'$, and gas-exchange traits; the third one is related to circadian period. PCA and structural equation modeling revealed the correlation structure of complex traits and potential mechanistic relationships, including how circadian period both directly and indirectly interacts with and might influence physiological trait expression (Fig. 5; Fig. 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.

**Acknowledgements**

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**References**


significant effects on leaf-to-canopy gas exchange under field conditions. bioRxiv, 054593.


responsible for naturally occurring variation in circadian period in Brassica rapa. 

Proceedings of the National Academy of Sciences, 112, 3829-3834.


Figure 1. Differences in quantum yield of PSII ($F_{v'}/F_{m'}$) (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 ($p < 0.05$).
Figure 2. Association between circadian period and quantum yield of photosystem II (Fv/Fm') at different light levels.

(a) Association between circadian period and Fv'/Fm' 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: R²=0.19, p=0.01

(b) Association between circadian period and Fv'/Fm' 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: R²=0.53, p=0.0099

(c) Association between circadian period and Fv'/Fm' 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: R²=0.38, p=0.03
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 ($q_E$, $q_T$ and $q_I$) 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) $R^2 = 0.69$, $p = 0.02$

(b) $R^2 = 0.38$, $p = 0.04$
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: R²=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: R²=0.38, p=0.04
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 $NPQ$.

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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.

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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

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