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Mutations that alter Arabidopsis flavonoid metabolism affect the circadian clock

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SUMMARY

Flavonoids are a well-known class of specialized metabolites that play key roles in plant development, reproduction, and survival. Flavonoids are also of considerable interest from the perspective of human health, as both phytonutrients and pharmaceuticals. RNA sequencing analysis of an Arabidopsis null allele for chalcone synthase (CHS), which catalyzes the first step in flavonoid metabolism, has uncovered evidence that these compounds influence the expression of genes associated with the plant circadian clock. Analysis of promoter-luciferase constructs further showed that the transcriptional activity of *CCA1* and *TOC1*, two key clock genes, is altered in CHS-deficient seedlings across the day/night cycle. Similar findings for a mutant line lacking flavonoid 3′-hydroxylase (F3′H) activity, and thus able to synthesize mono- but not dihydroxylated B-ring flavonoids, suggests that the latter are at least partially responsible; this was further supported by the ability of quercetin to enhance *CCA1* promoter activity in wild-type and CHS-deficient seedlings. The effects of flavonoids on circadian function were also reflected in photosynthetic activity, with chlorophyll cycling abolished in CHS- and F3′H-deficient plants. Remarkably, the same phenotype was exhibited by plants with artificially high flavonoid levels, indicating that neither the antioxidant potential nor the light-screening properties of flavonoids contribute to optimal clock function, as has recently also been demonstrated in animal systems. Collectively, the current experiments point to a previously unknown connection between flavonoids and circadian cycling in plants and open the way to better understanding of the molecular basis of flavonoid action.

Keywords: ***Arabidopsis thaliana*, *CCA1*, chlorophyll, circadian clock, flavonoids, luciferase, quercetin, *tt4*, *tt7*, *TOC1*.**

INTRODUCTION

Plants produce an astonishing array of natural products via complex networks of specialized metabolism (Fernie & Tohge, 2017). These unique biochemicals, which number in the hundreds of thousands across the plant kingdom, are essential for plant growth, survival, and reproduction, and are critical mediators of interactions with other organisms. Flavonoids are one of the major groups of specialized metabolites and are present in virtually every member of the plant kingdom. The biosynthetic pathway present in extant plant species has been traced back to the emergence of plants onto land some 550–470 million years ago and to have been key to the eventual colonization of virtually every terrestrial niche (Davies et al., 2020; Yonekura-Sakakibara et al., 2019). It has been hypothesized that flavonoids initially functioned as physiological regulators and chemical messengers, consistent with the presumed very low concentrations and simple structures of the products produced by the ancestral pathway (Stafford, 1991). This system evolved to enable the production of the multiple classes and more than 9000 different structures documented to date with diverse roles that include mediating phytohormone activity and reproduction, defense from predators and pathogens, and protection from heavy metals, UV light, and other stresses. Flavonoids also play key roles in positive interactions with other organisms, including in recruiting pollinators and modulating the plant microbiome, with activities in animals making flavonoids of considerable interest as phytonutrients and pharmaceuticals (Crozier et al., 2009; Guo et al., 2019; Maher, 2019; Wang et al., 2018).

Plant flavonoid biosynthesis is carried out by highly conserved machinery, with the first two steps catalyzed by enzymes derived from fatty acid metabolism and subsequent reactions by members of more broadly dispersed enzyme superfamilies (Yonekura-Sakakibara et al., 2019). This system is organized as a multienzyme complex, which in at least some cases appears to be assembled around the entry-point enzyme, the type III polyketide synthase chalcone synthase (CHS) (Nakayama et al., 2019), with competitive interactions controlling the distribution of flux into branch pathways (Crosby et al., 2011). This enzyme complex is localized, in part, at the cytoplasmic face of the endoplasmic reticulum, with the resulting end products (flavonols, anthocyanins, and proanthocyanidins in Arabidopsis) transported primarily to the vacuole and cell wall (Agati et al., 2012; Zhao, 2015). Flavonoid enzymes have also been reported to localize to the nucleus in numerous species, where they may be responsible for the synthesis of flavonoids *in situ* (Winkel, 2019; Zhang et al., 2020). These proteins may have alternative functions, as suggested by our report that CHS interacts with MOS9, a nuclear protein required for defense gene expression with no known function in flavonoid metabolism (Watkinson et al., 2018).

To explore CHS function from a broader perspective, RNA sequencing (RNA-Seq) was used to examine the effects of a null mutation in Arabidopsis CHS (*tt4-11*; Figure 1a) on global gene expression. Surprisingly, this analysis uncovered alterations in the expression of the central circadian clock genes as well as a large number of clock-regulated genes. These findings were corroborated in complementary experiments, including showing effects at the level of transcriptional control of the core clock genes, *CLOCK ASSOCIATED 1* (*CCA1*) and *TIMING OF CAB EXPRESSION 1* (*TOC1*), as well as disruption of cycling of photosynthetic activity, an established indicator of endogenous circadian rhythms. Analysis of a *tt7* mutant line, which is deficient in flavonoid 3′-hydroxylase (F3′H) activity (Figure 1a), suggests that dihydroxylated flavonoids may be responsible for the observed effects of flavonoids on clock function. It has been well established that flavonoid gene expression and the accumulation of pathway end products fluctuate across the day/night cycle and that this is driven at least in part by interaction of the clock

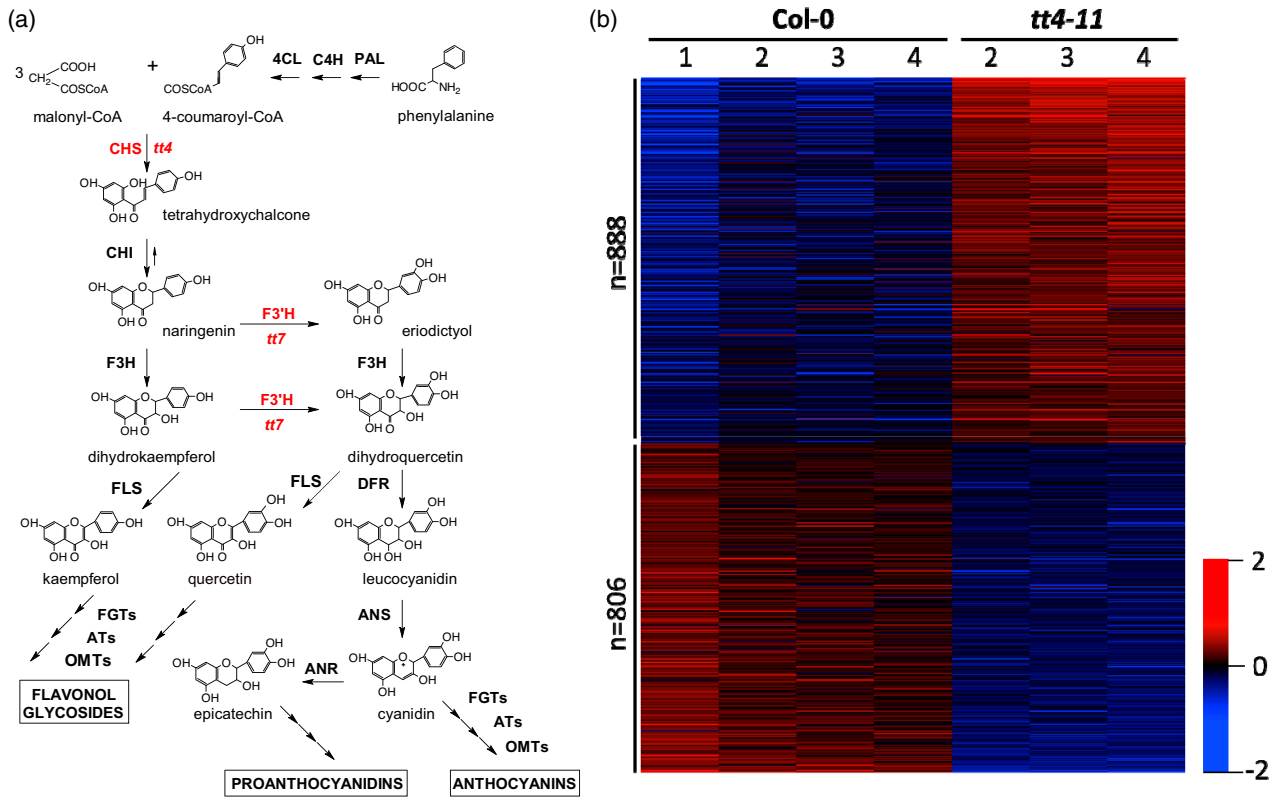

Figure 1. Effects of disruption of flavonoid metabolism on the seedling transcriptome.
(a) Simplified schematic of the Arabidopsis flavonoid pathway showing locations of the enzymatic steps disrupted in *tt4-11* and *tt7-5*. The two enzymes and corresponding loci that are the focus of this study are highlighted in red text. Boxed text identifies the three major flavonoid end products in Arabidopsis. Enzyme names are abbreviated as follows: phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), flavonoid glucosyl transferase (FGT), acyl transferase (AT), and *O*-methyl transferase (OMT). (b) Heatmap of z-scores for DEGs in Col-0 samples 1–4 and *tt4-11* samples 1–3 meeting the criteria $|\log_2(\text{fold change})| \geq 0.50$ and $P \leq 0.05$. Color scale: red = elevated and blue = reduced in *tt4-11*.

transcription factor CCA1 with flavonoid gene promoters (Harmer et al., 2000; Liebelt et al., 2019; Nagel et al., 2015; Soengas et al., 2018). This study provides the first evidence for a connection between flavonoids and plant clock function and opens the doors to a better understanding of the molecular basis of flavonoid action in a myriad of cellular processes.

RESULTS

CHS-deficient lines exhibit altered expression of numerous clock-associated genes

While the primary site of flavonoid metabolism in cells has long been held to be the cytoplasmic face of the endoplasmic reticulum, a number of studies in recent years point to the nucleus as another location of active flavonoid biosynthesis as well as potential moonlighting roles for the enzymes (Winkel, 2019; Zhang et al., 2020). This raises the possibility that flavonoid enzymes or end products may directly or indirectly influence gene expression. To examine this further, we undertook transcriptome analysis of a well-characterized Arabidopsis flavonoid null mutant, *tt4-11* (Lewis et al., 2011). This allele carries a T-DNA insertion in the second exon of the CHS gene, resulting in loss of all detectable CHS protein and flavonoid end products (Bowerman et al., 2012; Lewis et al., 2011). RNA-Seq was used to compare the transcriptomes of wild-type and *tt4-11* seedlings grown under conditions of maximal flavonoid accumulation, consisting of 6 days of germination on 1× MS with 2% sucrose under continuous white light illumination (LL) (Dataset S1). This analysis revealed just 163 genes with differences in expression meeting a stringent criterion of $|\log_2(\text{fold change})| \geq 1$ (i.e., fold change ≥ 2 or fold change ≤ 0.5), all with *P*-values of <<0.001 (Dataset S2). Of these differentially expressed genes (DEGs), 121 had elevated transcript levels in *tt4-11* and 42 (besides *CHS*) had levels that were reduced. Many more genes met the more conventional cutoff of $|\log_2(\text{fold change})| \geq 0.5$ (i.e., fold change ≥ 1.41 or fold change ≤ 0.71) and $P \leq 0.05$: 1694 in total, with 888 upregulated and 806 downregulated (not including *CHS*) in the mutant line (Figure 1b; Dataset S3).

The 163 high-confidence DEGs encompassed several functional groups containing multiple members. These included 12 genes encoding ubiquitin ligases and 17 genes involved in ethylene response or metabolism with elevated expression in *tt4-11* (1.4- to 5.1-fold), as well as a number of genes encoding transporters and protein kinases with both elevated and reduced transcript levels (-1.7 to 3.7-fold). Unexpectedly, one of the largest functional groups (10 of the 163 high-confidence DEGs) encoded components of the core circadian clock and clock-associated proteins (Figure 2). This was also reflected in the PANTHER GO-Slim Biological Process analysis (Mi et al., 2020), with the highest

Gene Symbol	Gene name	log2Fold Change	p-value
CORE CLOCK			
CCA1	AT2G46830.1	2.1283	6.96E-28
CCA1	AT2G46830.2	1.5956	7.66E-10
LNK2	AT3G54500.3	1.2121	2.29E-06
RVE8	AT3G09600.1	1.0339	6.00E-05
RVE2	AT5G37260.1	0.3685	0.0200
ELF3	AT2G25930.1	-0.3349	0.0323
PRR7	AT5G02810.1	-0.4250	0.0196
TOC1	AT5G61380.1	-0.4918	1.79E-04
PRR5	AT5G24470.1	-0.7037	2.52E-03
BOA	AT5G59570.1	-0.8615	8.76E-04
ELF4	AT2G40080.1	-1.1359	6.44E-11
CLOCK-ASSOCIATED			
LNK3	AT3G12320.1	1.4038	1.53E-08
REV3	AT1G01520.1	1.1400	2.81E-06
CCL1	AT5G65480.1	0.3994	5.64E-03
CRY1	AT4G08920.1	0.3248	5.90E-03
NUP205	AT5G51200.1	0.3209	9.09E-03
CKA2	AT3G50000.1	0.2945	0.0141
CKA1	AT5G67380.1	-0.6177	0.0361
ARR4	AT1G10470.1	-0.7879	1.03E-07
GI/FB	AT1G22770	-1.0197	6.50E-08
ADO3	AT1G68050.1	-1.6202	1.89E-11
CCL	AT3G26740.1	-1.8140	9.35E-36

Figure 2. Clock genes with altered transcript levels in CHS/flavonoid-deficient Arabidopsis seedlings.
Positive values indicate elevated expression in *tt4-11* relative to wild type. Color scale: red = elevated and blue = reduced in the mutant line. Genes not meeting the $|\log_2(\text{fold change})| \geq 0.5$ cutoff are shown in gray font.

enrichment (44.29-fold) for genes associated with circadian rhythms (Dataset S4). In fact, transcript levels for the key circadian transcriptional regulator, CCA1, were the third-most elevated of any gene in the dataset ($\log_2$(fold change) = 2.13, i.e., 4.4 fold), while transcripts for the clock-associated gene *CCL* were the most reduced ($\log_2$(fold change) = -1.81, i.e., -3.5-fold). The high-confidence DEGs also exhibited a substantial (almost 2-fold) enrichment of predicted circadian-regulated genes, with 60% belonging to this group in *tt4-11* (64 of 106 expressed genes) (Dataset S2) compared to approximately one third of the wild-type circadian transcriptome (Covington et al., 2008). Consistent with this finding, a large number of the high-confidence DEGs contained

CCA1 binding sites previously identified by ChIP-Seq (Dataset S3; Nagel et al., 2015): 16 (13%) of those with elevated transcript levels in *tt4-11* and 27 (64%) of those with reduced levels. It is also worth noting that members of the functional classes mentioned above have associations with clock control: two of the five ubiquitin ligases with elevated levels in *tt4-11* are among those identified as potential regulators of circadian function by Feke et al. (2019); one of the ethylene response factors (ERFs), ERF18, has CCA1 binding sites in its promoter and could participate in modulation of the clock by ethylene (Haydon et al., 2017); and one member of the glycine-rich RNA-binding protein group, AtGRP7, has been shown to regulate the circadian cycling of its own transcript (Staiger & Heintzen, 1999). Many more core clock and clock-associated transcripts met the more conventional $|\log_2(\text{fold change})| \geq 0.5$ cutoff with $P \leq 0.05$, including virtually all components of the Arabidopsis clock machinery (Singh & Mas, 2018). This larger group of DEGs was also enriched for predicted circadian-regulated genes (46% compared to 32% for all genes in the Covington/Edwards collection; Dataset S3), although not quite as much as the high-confidence DEGs (at 60%).

To validate the results of the transcriptome experiment and further explore these initial findings, qPCR was used to compare the expression of several of the clock-associated genes under both LL and 12 h/12 h light/dark (LD) conditions. For this analysis, comparisons were made in *tt4-11* and Col-0 seedlings, as well as in another CHS null allele, *tt4-2* (Bennett et al., 2006; Burbulis et al., 1996). As illustrated in Figure 3, the differences detected by qPCR were smaller than observed by RNA-Seq and statistically significant in only some cases, due in part to the relatively low levels of expression of these genes, particularly in LL. Overall, however, the expression patterns in seedlings entrained to LL mirrored those observed in the transcriptome dataset in both *tt4* alleles, thereby also confirming that the observed effects are linked to the *CHS* locus (Figure 3a). Surprisingly, the opposite effect on clock gene expression was observed in seedlings entrained to LD, at both the morning and evening timepoints (Figure 3b, a.m. and p.m., respectively). The differential effects under LD and LL conditions may reflect a further role for flavonoids in the response to light stress that is overlaid on the contribution to circadian rhythmicity.

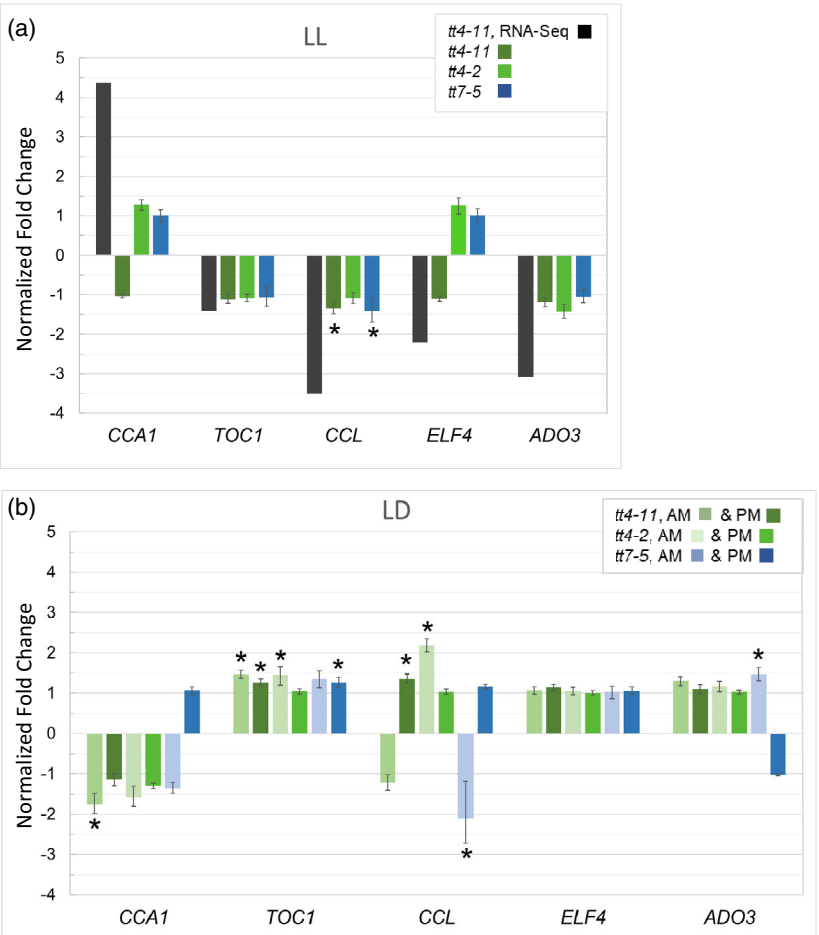

Figure 3. qRT-PCR analysis of select clock-associated genes in flavonoid mutant lines.
Differences are shown as fold change in gene expression in mutant lines relative to the corresponding wild type; error bars are standard errors ($n = 3$ biological replicates), with asterisks indicating a significant difference ($P < 0.05$) from the corresponding wild type per Student's *t*-test. (a) Seedlings grown in continuous light (LL); RNA-Seq data converted to fold change values are shown for comparison. (b) Seedlings entrained in 12 h/12 h LD with samples collected at 4 h (a.m.) and 12 h (p.m.) after lights came on.

The *tt4-11* allele alters *CCA1* and *TOC1* promoter activity *in vivo*

To determine whether the changes in steady-state mRNA levels observed in *tt4* might originate at the transcriptional level, we took advantage of Arabidopsis lines containing the promoter-luciferase (LUC) constructs *CCA1p::LUC* or *TOC1p::LUC* (Salomé & McClung, 2005). These lines have been used extensively to monitor circadian transcriptional activity in intact plants in real time. The two promoters exhibit complementary patterns of expression, with *CCA1* maximal in the morning and *TOC1* in the evening, approximately 12 h out of phase. The two constructs were introduced into *tt4-11* by crossing with the wild-type Col parental lines, followed by identification of multiple independent F3 lines homozygous for *tt4-11* and expressing the transgene. Seedlings were germinated on MS-sucrose plates for 5 days under 12 h/12 h LD conditions and then moved to constant darkness (DD) and LUC activity was monitored for approximately 10 days. The resulting traces showed that *CCA1* and *TOC1* promoter activity mirrored transcript levels (Figure 3b), with expression elevated and reduced, respectively, in *tt4-11* relative to wild-type Col-0 seedlings across the day/night cycle (Figure 4a).

The data were detrended (baseline-subtracted) and statistical analysis of amplitude, phase, and period was performed using BioDare2 (Figure 4b,c; Figure S1). This revealed significant changes in amplitude for *TOC1* in both mutant backgrounds, consistent with the role of this transcription factor in mediating clock function in response to a wide range of conditions, including abiotic factors such as drought (Legnaioli et al., 2009), temperature (Zhu et al., 2016), and light (Soy et al., 2016). There were also modest effects on period and phase in *tt4-11* (Figure S1). In contrast, little effect was observed on the amplitude, phase, or period of *CCA1* promoter activity pre-conditioned in LD (Figure S1), reflecting the results of a study by Pruneda-Paz et al. (2014) that failed to identify transcription factors mediating *CCA1* amplitude. These findings indicate that the effects of flavonoids on the plant clock under LD conditions occur, at least in part, at the level of transcription, particularly in the case of *TOC1*, as well as through post-transcriptional processes in the case of *CCA1*.

A parallel analysis performed with seedlings entrained to LL conditions showed that *CCA1* and *TOC1* promoter activity was substantially lower overall in LL than in LD (top and middle panels versus bottom panel, Figure 4a). This is consistent with the overall lower steady-state RNA levels observed by qPCR. However, relative promoter activities in the mutant and wild-type lines were the same as in LD, and therefore opposite the trends observed for steady-state transcripts, where the levels of *CCA1* were higher and those of *TOC1* were lower in LL (Figures 2 and 3). The same pattern was displayed in the relative amplitudes of the detrended traces, with *CCA1* having an apparent reduction and *TOC1* an apparent elevation, although only the latter was statistically significant due to the low and variable amplitudes of the rhythms in LL (Figure 4b,c). This suggests that if flavonoids do play an additional role in seedlings under light stress, this may be achieved via post-transcriptional control.

The effect of the *tt4-11* mutation on clock activity is evident in photosynthetic rhythmicity

To determine whether the effects of flavonoids on clock gene expression translated to physiological outputs, we assessed the cycling of photosynthetic activity, an established indicator of endogenous clock function (Dodd et al., 2015; Guadagno et al., 2018; Shor et al., 2017). For this analysis, we first used hyperspectral imaging, which has been shown to provide an effective real-time, non-destructive measure of photosynthetic activity in individual soybean (*Glycine max*) and wheat (*Triticum aestivum*) leaves (Pan et al., 2015). To test this in Arabidopsis, Col-0 and *tt4-11* plants were grown on soil for 5–6 weeks in 12 h/12 h LD and then transferred to LL, and leaf reflectance was measured from 385 to 1027 nm. This analysis uncovered a strong diurnal periodicity in three of five Col-0 plants examined over a 48-h period at both 659 nm (Figure 5a) and 469 nm (Figure S2), the optimal wavelengths for detecting chlorophyll *a* and *b*. In contrast, no periodicity was observed in any of the five *tt4-11* plants that were analyzed.

This distinction was even more evident in a spectrophotometric analysis of chlorophyll content in 4.5-week-old soil-grown plants. Using this more conventional approach (Barnes et al., 1992), *tt4-11* plants exhibited no evidence of cycling of chlorophyll *a* and *b* levels in contrast to the strong periodicity apparent in Col-0 plants (Figure 5b). These differences show that disruption of core clock and clock-regulated genes associated with the loss of CHS and/or flavonoids is reflected in alterations in clock-controlled physiology in these plants.

Disruption of the dihydroxyflavonoid branch pathway alone also affects clock gene expression

To determine whether it was the absence of CHS protein or of specific flavonoids that was responsible for the observed effects on clock gene expression, we examined a null allele for *tt7*, the locus for the single-copy F3′H gene in Arabidopsis. Disruption of this gene affects one half of the central pathway, disabling the synthesis of quercetin and its derivatives, one of two abundant and ubiquitous flavonols present in Arabidopsis. It also disrupts the synthesis of cyanidin and epicatechin, precursors of the primary anthocyanin and proanthocyanidin pigments present in this species (Figure 1a). This alteration results in somewhat elevated levels of kaempferol, but does not affect the accumulation of sinapate esters, as has been observed in

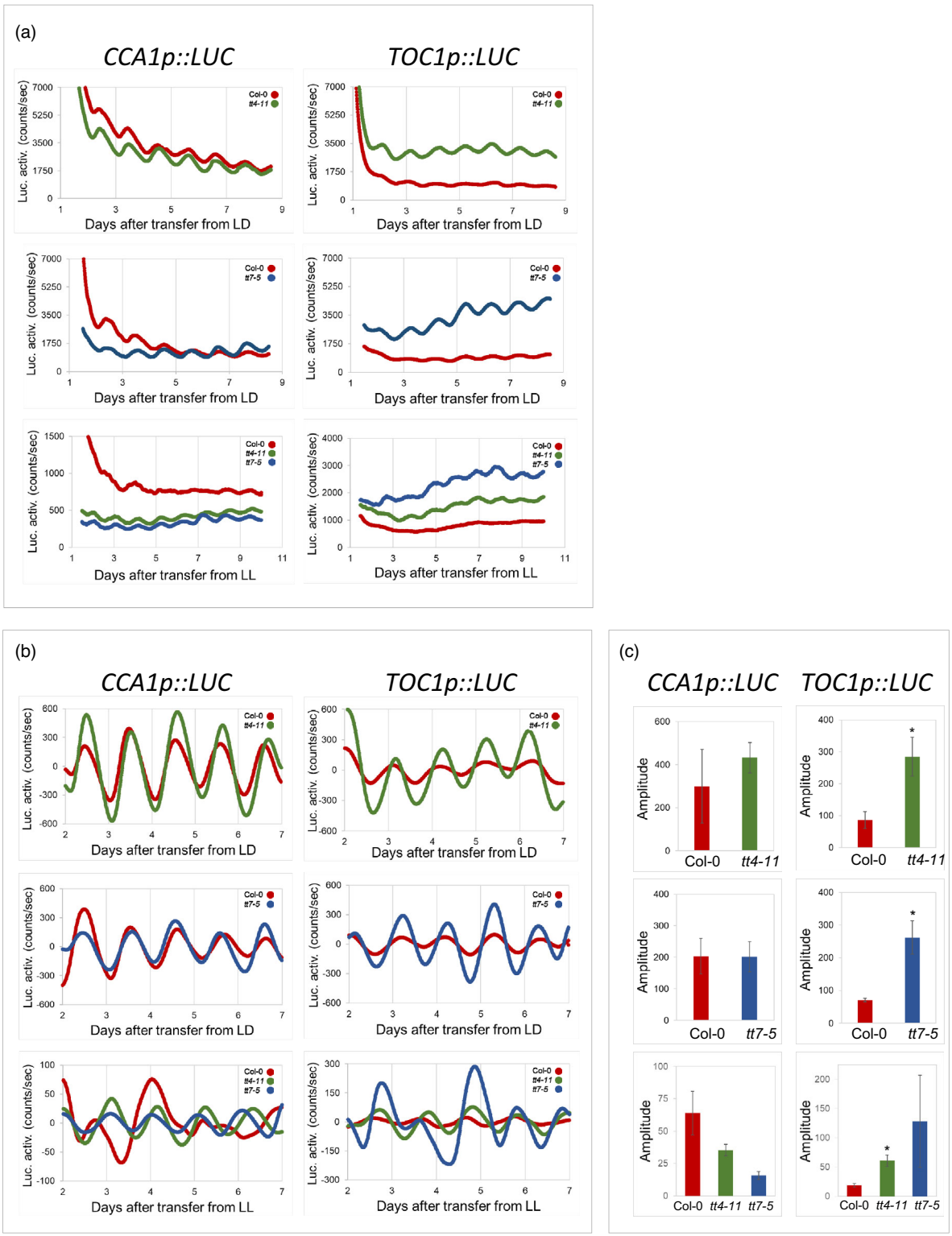

Figure 4. Effects of flavonoid mutations on *CCA1* and *TOC1* promoter activity.
Luciferase activity was assessed in seedlings following transfer from 12 h/12 h LD (top two rows) or LL (bottom row) to continuous darkness (DD) using a Lumicycle luminometer. In all panels, results are shown for wild-type (red), *tt4-11* (green), and *tt7-5* (blue) seedlings. Each line represents the average of four biological replicates for wild type and the average of two to four replicates for each of three independent F3 lines for *tt4-11* and *tt7-5*. (a) Unprocessed and (b) detrended (baseline-subtracted) data. (c) Comparison of amplitudes of detrended data. Error bars are standard error. $^{*}P < 0.05$, Student's *t*-test.

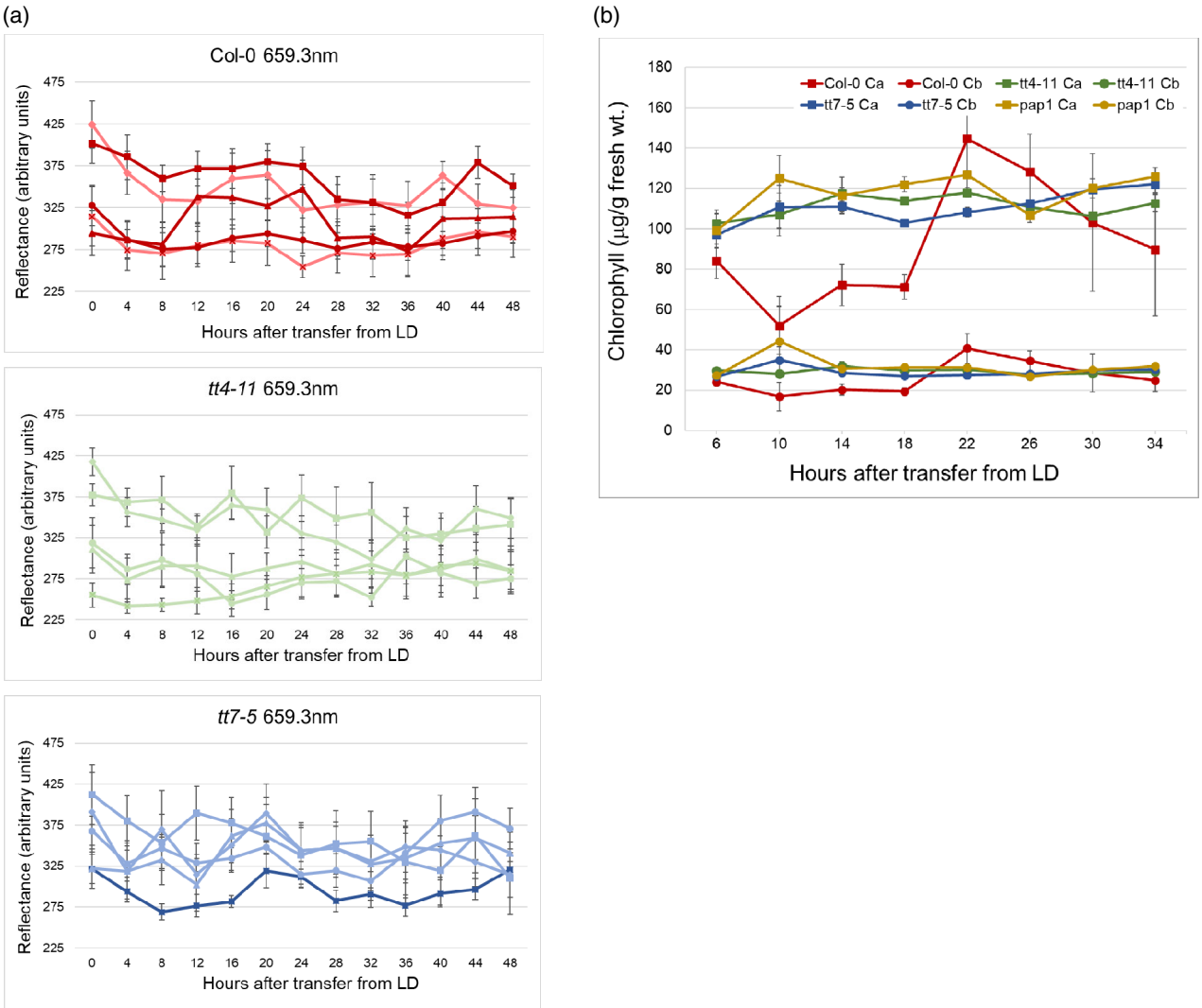

Figure 5. Effects of flavonoid mutations on cycling of chlorophyll levels in Arabidopsis plants.
Plants were entrained in 12 h/12 h LD and transferred to LL before data or sample collection. (a) Hyperspectral imaging at the optimal wavelength for chlorophyll *a* (470 nm) and *b* (660 nm); lines represent the average reflectance of nine leaves from each of five 5–6-week-old plants tracked for 48 h after shifting from LD to LL. Nine leaves were analyzed on each of five plants for each genotype. Dark lines represent plants exhibiting diurnal rhythmicity with $P < 0.05$ per metacycle analysis. (b) Spectrophotometric quantification of intracellular chlorophyll *a* and *b* content in above-ground parts of 4.5-week-old plants. Data points represent the average value calculated from three to four plants at each time point. Error bars in all panels are standard error.

tt4 (Bowerman et al., 2012; Li et al., 1993; Stracke et al., 2007).

Analysis of the F3′H null allele *tt7-5* (Bowerman et al., 2012) uncovered the same alterations in *CCA1* and *TOC1* promoter activity as we observed for *tt4-11* (Figure 4). Effects on steady-state mRNA levels were also similar, although again not always statistically significant (Figure 3). Importantly, *tt7-5* plants lacked photosynthetic rhythmicity as assessed by both hyperspectral imaging and extracted chlorophyll *a* and *b* levels (Figure 5) Together, these results indicate that it is not lack of CHS protein or elevated levels of sinapate esters, but products of the flavonoid pathway and dihydroxylated flavonoids such as quercetin, cyanidin, and their derivatives in particular that may be responsible for the observed effects of the *tt* mutants on clock function.

To examine this further, we explored the possibility that supplying quercetin in the growth medium alters *CCA1p::LUC* expression in seedlings. Previous work has established good evidence for the uptake and transport of exogenous flavonoids, including quercetin, from the growth medium by Arabidopsis plants (Buer et al., 2007). In this experiment, entrainment of wild-type seedlings in 12 h/12 h LD on MS-sucrose medium containing 1 µM quercetin enhanced *CCA1* promoter activity relative to control seedlings (Figure 6). Quercetin also elevated *CCA1*

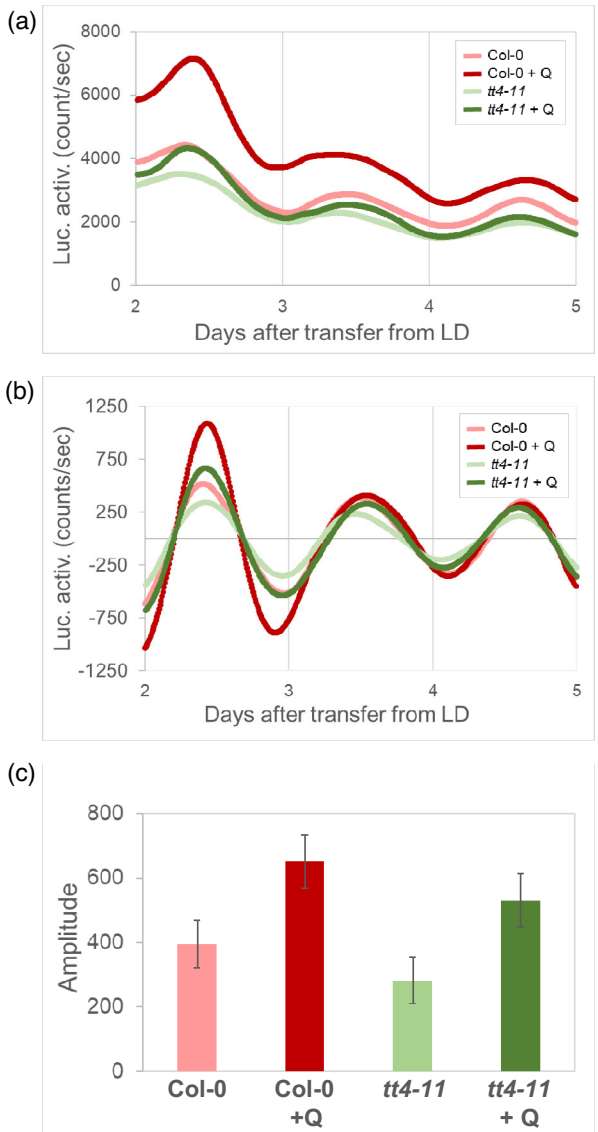

Figure 6. Effects of quercetin on *CCA1p::LUC* expression in wild type and *tt4-11*.
Seedlings were grown on MS-sucrose medium in the presence of 1 μM quercetin or an equal volume of solvent. Luciferase activity was assessed after 6 days of growth in 12 h/12 h LD as described for Figure 4, with three to four plates of 14 seedlings per condition. (a) Unprocessed and (b) detrended (baseline-subtracted) data. (c) Comparison of amplitudes of detrended data. Error bars are standard error. Student's *t*-tests comparing control and treated samples gave a *P*-value of 0.06 for wild type and 0.08 for *tt4-11*.

promoter activity in *tt4-11* to levels that appeared similar to those in wild-type seedlings. This finding suggests that quercetin and/or its derivatives could be active players in modulating rhythmicity in Arabidopsis.

As a complementary assessment of the effects of elevated levels of flavonoids on rhythmic processes, we examined chlorophyll cycling in *pap1* plants, which accumulate high endogenous levels of flavonoids due to ectopic overexpression of a *MYB* transcription factor (Borevitz et al., 2000). Surprisingly, these seedlings lacked rhythmic chlorophyll levels, similar to *tt4-11* and *tt7-5* (Figure 5b), suggesting that there could be an optimal level at which flavonoids influence the output of clock activity in Arabidopsis. This lack of cycling in the presence of elevated levels of flavonoids further indicates that it is neither the antioxidant potential nor the light-screening properties of these compounds that is responsible for the observed disruption of clock function.

DISCUSSION

Virtually every living organism possesses a clock mechanism that coordinates its biological processes with the rhythmic day/night cycle of light and temperature. However, the clock machinery has evolved independently in the different eukaryotic lineages so that the plant, insect, and animal clocks have few components in common, despite being organized around similar design principles. The plant clock machinery is particularly complex, reflecting its integration with response pathways for a wide range of environmental signals, from light and temperature to biotic and abiotic stress (Bendix et al., 2015; Creux & Harmer, 2019; McClung, 2019). Perhaps not surprisingly, the clock contributes to both plant resilience and productivity, and selection of circadian gene variants appears to have been key to the domestication of agriculturally important plant species, affecting both flowering time and adaptation to stress (Creux & Harmer, 2019). As in all eukaryotes, the core clock is characterized by an interlocked transcription–translation feedback loop (TTFL), which in plants is centered on CCA1 and TOC1 as core components of the morning and evening clocks. The TTFL orchestrates the expression of a vast array of downstream genes and is itself regulated by a growing list of small molecules and hormones at both the transcriptional and post-transcriptional levels (Hearn & Webb, 2020). Signaling by reactive oxygen species (ROS) is emerging as a key element in a feedback system that coordinates the core circadian machinery with a wide variety of rhythmic cellular processes, including photosynthesis in plants (Guadagno et al., 2018; Simon et al., 2019).

Global analysis of gene expression in Arabidopsis *tt4* mutants has uncovered new evidence for effects on the plant circadian clock involving the abundant and ubiquitous phytochemicals known as flavonoids. Transcriptome, qPCR, and *in vivo* promoter analyses in flavonoid-deficient *tt4* seedlings indicate that clock gene expression is altered by disruption of the *CHS* locus. Similar observations for the F3′H null allele *tt7-5* suggest that dihydroxy B-ring flavonoids are a contributing factor. These conclusions are further supported by the finding that *tt4-11* and *tt7-5* plants exhibit marked defects in the periodicity of photosynthetic activity and that growth on quercetin-containing medium

enhances expression of *CCA1p::LUC* in both wild-type and *tt4-11* seedlings. Unexpectedly, *pap1* seedlings were also found to lack chlorophyll rhythmicity, indicating that normal clock activity may rely, directly or indirectly, on an optimal intracellular level of flavonoids.

Prior to the current study, there was just one publicly available transcriptome dataset for a flavonoid-deficient plant line, used to examine the contribution of flavonoids to oxidative and drought tolerance (Nakabayashi et al., 2014). These experiments used a different allele of *tt4* (*tt4-3*, which is also a null allele in a Columbia background; Shikazono et al., 1998) and mature plants grown on 1% sucrose under 16 h/8 h LD conditions (versus 6-day-old seedlings, 2% sucrose, LL in our case). Our re-examination of this dataset using Genevestigator (Hruz et al., 2008) identified alterations in the expression of numerous core clock and clock-associated genes, echoing the results of our study. However, the effects were consistently in the opposite direction (Dataset S5), the same as we observed by qPCR in LD-entrained seedlings (Figure 3). Genes not associated with the clock or not under circadian control generally did not exhibit this inverse relationship (Dataset S5). These microarray data thus provide further evidence that flavonoids contribute circadian rhythmicity and do so somewhat differently under LL and LD conditions. The data also show that *tt4* mutations affect gene expression, not only in seedlings, but also in mature plants.

Interestingly, the WD-repeat protein TTG1 was recently shown to be capable of mediating the plant circadian clock (Airoldi et al., 2019). This unique transcriptional regulator controls not only trichome development and the production of seed coat mucilage, but also the synthesis of anthocyanins and proanthocyanidins (Figure 1a). However, the effect of TTG1 on the plant clock was attributed in that study, not to any of these activities, but rather to retention of functionality present in a common ancestor of TTG1 and the related LIGHT-REGULATED WD1 and LIGHT-REGULATED WD2 clock regulators. Our finding that flavonoids themselves may impact clock function suggests that there may be additional interpretations of these results.

Despite decades of research on the physiological effects of flavonoids in plants, understanding of the underlying modes of action remains remarkably murky (Agati et al., 2020; Winkel, 2019). Flavonoid bioactivity has invariably been ascribed to the strong antioxidant potential of these compounds, which can be present at quite high levels in certain tissues and in response to diverse biotic and abiotic stresses, although this remains a matter of substantial debate (Agati et al., 2020). There is good evidence that flavonoids do function as antioxidants in some situations, for example, in glandular trichomes, where lack of flavonoids increases production of ROS (Sugimoto et al., 2021), and in protecting plant tissues from oxidative stress associated with drought or high temperatures (Muhlemann et al., 2018; Nakabayashi et al., 2014). There is also a strong connection between the clock and the intracellular redox state in plants, which is modulated both by endogenous metabolic processes, including respiration and photosynthesis, and by environmental conditions that elevate intracellular ROS (Guadagno et al., 2018). It is therefore reasonable to suppose that flavonoids may contribute to maintaining ROS homeostasis and the integrity of the plant clock; a reduction in antioxidant potential could lie behind the observed disruption of clock gene expression and cycling of chlorophyll levels in the *tt4* and *tt7* lines.

One prediction of the hypothesis that the antioxidant potential of flavonoids contributes to optimal clock function is that the *tt4* transcriptome should display the hallmarks of oxidative stress. To examine this possibility we compared the high-confidence DEGs for *tt4-11* with the 'common stress transcriptome', 197 genes identified by analysis of publicly available microarray data as being induced by a wide variety of biotic and abiotic stressors (Ma & Bohnert, 2007). This analysis showed that 25 of the 120 high-confidence upregulated genes in the *tt4-11* transcriptome were members of this group (Dataset S2), including the gene that was most highly induced, At1g74450. Although not yet functionally characterized, this gene has been shown to provide a link between multiple environmental stresses and plant resilience, including pollen development (Visscher et al., 2015). Overall, the *tt4-11* expression profile suggests that the absence of flavonoids does indeed result in a persistent, if modest, stress condition in LL-grown seedlings.

To examine whether this response is related specifically to a lack of antioxidant potential in *tt4-11*, the high-confidence list was further compared to the genes shown by Gadjev et al. (2006) to exhibit altered expression in ROS-sensitive Arabidopsis lines. However, at most three to four of the multiple genes defined in mutant lines deficient in the response to either singlet oxygen, superoxide, or H_2O_2 were also mis-expressed in *tt4-11*, and in many cases showed the opposite change, being induced rather than suppressed and *vice versa* (Dataset S2). This low number was somewhat surprising considering that many ROS-associated processes are rhythmic, but is consistent with only a subset of circadian-controlled genes having altered expression in the *tt4-11* transcriptome. The *tt4-11* DEGs did include the mitogen-activated protein kinase (MAPK) kinase kinase gene *MAPKKK14* ($\log_2$(fold change) = 1.329, i.e., 2.51-fold), but no other components of the stress response-associated MAPK pathway. There was also modest elevation ($\log_2$(fold change) = 0.713, i.e., 1.64-fold) of *AGC2*, a gene that is strongly induced by singlet oxygen, the primary ROS associated with photooxidative damage in chloroplasts under excess light energy (Shumbe et al., 2016). In addition, the *CAT2* gene, which encodes

the predominant catalase critical for scavenging H_2O_2 in Arabidopsis (Mhamdi et al., 2010), exhibited elevated expression in the *tt4-11* LL transcriptome ($\log_2$(fold change) = 1.036, i.e., 2.05-fold). However, this enzyme is also tightly linked with control of the central clock machinery in plants, as well as other organisms (Shim & Imaizumi, 2015). Expression of *AOX1a*, which encodes mitochondrial alternative oxidase, an important indicator of the response to excessive ROS, was unchanged and only two peroxiredoxin-like genes, neither of which has been linked to circadian sensing, exhibited small changes (one up and one down) in transcript levels (Lee et al., 2018). Overall, the *tt4-11* DEG set gave little support for the hypothesis that these fully flavonoid-deficient seedlings were suffering from a lack of antioxidant capacity. This is in spite of good evidence that the converse situation, overaccumulation of flavonoids in mutant lines or due to exogenous application, can protect plants from oxidative stress (Kurepa et al., 2016; Nakabayashi et al., 2014). It may also be evidenced by the finding that *pap1* seedlings, with severely elevated endogenous levels of flavonoids, exhibited similarly arrhythmic chlorophyll levels as the flavonoid-deficient lines (Figure 5).

An alternative possibility that is receiving growing interest is a potential role for phenylpropanoids, in general, as a sink for the diversion of excess carbon from primary metabolism. Activation of phenylpropanoid biosynthesis resulting in enhanced accumulation of lignin and anthocyanins, together with downregulation of various branch pathways of primary metabolism, is a well-documented response to pathogen attack, nutrient deprivation, and exposure to cold or transition metals (Arnold et al., 2012; Caretto et al., 2015; Hernández & Van Breusegem, 2010). This redirection of carbon flux alters the physical and chemical properties of plant cells and accompanies an overall reduction in plant metabolism and growth. Mutations in flavonoid genes have in several cases been found to alter flux within the flavonoid and sinapate branch pathways, although not into lignin biosynthesis (Li et al., 1993; Liu et al., 2002; Zhang et al., 2016). Thus one explanation for the observed effects of *tt4* and *tt7* on clock activity and photosynthetic cycling could be loss of an important sink for diurnal regulation of carbon flux, or alternatively, loss of a key metabolic signature for the integration of metabolic status and clock function. Neither is well supported by the current study, with altered photosynthetic cycling observed for all three mutant genotypes with altered flavonoid profiles, particularly *tt7*, which produces near-wild-type levels of many of these compounds (Bowerman et al., 2012; Stracke et al., 2007). Moreover, none of these lines exhibited observable alterations in plant growth relative to the wild type, either on sucrose-containing medium or on soil. There is also no evidence from the RNA-Seq data for *tt4-11* that lack of flavonoids alters expression of the phenylpropanoid, acetate, or sucrose biosynthetic pathways that supply carbon into flavonoid metabolism (Perez de Souza et al., 2020). However, GIGANTEA, a key component of the clock's sucrose signaling mechanism (Dalchau et al., 2011), is among the significantly reduced DEGs in the *tt4-11* RNA-Seq dataset (Dataset S2), suggesting that sucrose/carbon sensing could be impacted, directly or indirectly, in these plants.

A full understanding of the mechanisms underlying the influence of flavonoids, not only on circadian cycling, but also on a myriad of other cellular processes, will likely require a much better understanding of the interactions of these molecules with specific proteins. This idea has been gaining traction, particularly in animal systems where defining the mode of action of flavonoids is of considerable importance for drug development and efforts to enhance the human diet. In animals it has also long been argued that, other than in red blood cells and the intestine, concentrations are far too low for flavonoids to serve as antioxidants that provide protection against ROS (Crozier et al., 2009). Instead, the focus is increasingly on elucidating their interaction with components of intracellular signaling cascades, drug transporters, and metabolic enzymes, both *in vitro* and *in vivo* (Crozier et al., 2009; Gebicka, 2020; Middleton et al., 2000; Miron et al., 2017). Remarkably, it has recently been shown that a number of different flavonoids can modulate various aspects of the circadian clock in cultured animal cells and in mice (Xu & Lu, 2019). These studies provided evidence that alteration of circadian cycling by the polymethoxy flavone nobiletin involves binding to components of the ERK/CREB and REV-ERB/ROR signaling pathways (He et al., 2016; Shinozaki et al., 2017). Although homologous pathways are not present in plants, the component proteins are members of highly conserved families that occur in all eukaryotes, suggesting that common mechanisms of action may exist, even if these involve different specific targets. Although the identification of protein-binding targets of flavonoids in plants and other organisms lags well behind that in animals, there are a few notable examples, including direct binding and inhibition by quercetin of the plant-specific protein serine/threonine kinase PINOID, proposed to be the mechanism by which flavonoids control polar auxin transport (Henrichs et al., 2012). Defining the mechanistic basis of flavonoid action in plants through interaction with proteins and other biomolecules remains a fruitful area for further exploration.

The evidence for effects of flavonoids on circadian cycling is a new and intriguing example of the diverse and potent biochemical activities of plant specialized metabolites, both in plants and the organisms that consume them. Although the current study represents the first report for this association in plant systems, Hu et al. (2020) recently noted enhanced expression of the clock-associated genes

PRR5, *FT*, and *LHY* in two flavonoid-hyperaccumulating lines of licorice (*Glycyrrhiza glabra*), which suggests that the connection between flavonoids and the plant clock may well be a widespread phenomenon. The similar effects in animals, despite the dissimilarity of the clock machinery, raise the question of whether flavonoids also influence clock activity in yet other organisms, such as insects and microbes, and opens new doors to understanding the mode(s) of action of these potent and ubiquitous natural products in an important new context.

EXPERIMENTAL PROCEDURES

Plant genotypes and growth conditions

Flavonoid mutant lines *tt4-2* (back-crossed to wild type to remove a *max1* mutation in the background), *tt4-11* (SALK_020583), and *tt7-5* (SALK_053394) were described previously (Bennett et al., 2006; Bowerman et al., 2012; Burbulis et al., 1996; Lewis et al., 2011). Comparisons were made against Col-0 (CS7000) for *tt4-11* and *tt7-5*, or against a Columbia wild-type line originally obtained in Howard Goodman's laboratory at Harvard Medical School for *tt4-2*. The *CCA1p::LUC* and *TOC1p::LUC* promoter reporter lines in wild-type Col (Salomé & McClung, 2005) were a generous gift of Rob McClung (Dartmouth College). These were crossed with *tt4-11* and *tt7-5* using the mutant lines as the maternal parents, and F2 plants homozygous for the flavonoid gene mutation were selected based on the *transparent testa* phenotype. Multiple independent F3 lines were used for measurements of circadian parameters.

RNA-Seq and qRT-PCR

Gene expression analyses were performed using 5-day-old whole seedlings. Seeds were sown on 1× MS medium, pH 5.7, containing 2% sucrose and 0.8% agar as described previously (Kubasek et al., 1992), in 8- or 15-mm Petri dishes. Following stratification at 4°C in darkness for 3–5 days, seedlings were grown at 22°C in LL or a 12 h/12 h LD cycle (approximately 100 μE). At 5 days following germination, whole seedlings were harvested in 100-mg aliquots, flash frozen in liquid nitrogen, and stored at −80°C prior to processing. For RNA-Seq analysis, samples were collected within a 1-h time span, although the precise time of day was not recorded.

RNA was extracted using the Qiagen RNeasy Plant Maxi or Mini Kit with DNase on-column digestion (Qiagen, Germantown, MD, USA). For RNA-Seq analysis, libraries (four wild-type and four *tt4-11*) were prepared at the Virginia Tech Genomics Core Sequencing Laboratory using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina, San Diego, CA, USA), which uses polyA enrichment. These were sequenced on a HiSeq 2500 (Illumina) using Rapid Run 1×100 single read cycle clustering and sequencing. The resulting data were processed by the Virginia Tech Advanced Research Computing core facility as described in Chen et al. (2016). One of the *tt4-11* libraries was eliminated from the final analysis. Genes found to meet the cutoff for differential expression in *tt4-11* and wild type ($|\log_2(\text{fold change})| \geq 1.0$, i.e., ≥2-fold, all having *P*-values of <<0.001) were categorized based on function using the Bio-analytical Resource for Plant Biology's Classification SuperViewer (http://bar.utoronto.ca/). Genes related to clock function were curated manually. Microarray data (dataset AT-00697) described in Nakabayashi et al. (2014) were analyzed using Genevestigator (Hruz et al., 2008).

For quantitative real-time PCR (qRT-PCR) analysis, cDNA synthesis was carried out using SuperScript IV reverse transcriptase and RNaseOUT ribonuclease inhibitor (both from Invitrogen, Carlsbad, CA, USA) and 2 μg of total RNA for three biological replicates of each genotype (*tt4-2*, *tt4-11*, *tt7-5*, and wild type). qRT-PCR was performed using *Power* SYBR Green PCR Master Mix (Life Technologies Corp., Grand Island, NY, USA) using the primer pairs listed in Table S1. Analyses were carried out in 384-well plates in a QuantStudio 6Flex (Applied Biosystems, Bedford, MA, USA) with three technical replicates per sample. The resulting data were analyzed using the $\Delta\Delta C_T$ method and fold change values were normalized so that reduced expression was on a scale of -1 to -∞ (Livak & Schmittgen, 2001). Statistical analysis of C_T values was performed using JMP Pro 15.0.0. (SAS Institute Inc.).

Real-time bioluminescence measurements of promoter activity

Luciferase bioluminescence measurements were performed using seedlings containing *CCA1p::LUC* or *TOC1p::LUC* constructs. Growth was on MS-sucrose medium as described above, but using 35-mm dishes containing equivalent numbers (typically 5–15) of seedlings per dish. For quercetin feeding experiments, seedlings were grown on MS-sucrose-agar supplemented with 1 μM quercetin dihydrate (MP Biomedicals, Irvine, CA, USA) prepared using a 100 mM stock solution dissolved in dimethyl sulfoxide (DMSO); control seedlings were grown on medium containing an equivalent volume of the solvent. Following stratification for 2–3 days at 4°C in darkness, seedlings were germinated under a 12 h/12 h LD cycle or continuous light for 5 days. A 100-μl aliquot of 1 mM luciferin was added to the surface of each plate and reporter activity was recorded using a LumiCycle luminometer (Actimetrics, Wilmette, IL, USA) for up to 10 days. Detrended data were fit to a polynomial of three and smoothed over 30-min intervals using ClockLab (Actimetrics Inc.), and amplitude, phase, and period were quantified using FFT NLLS analysis in BioDare2 (Zielinski et al., 2014). Each experiment was repeated independently at least once.

Analysis of photosynthetic activity

Analyses were carried out on mature (5–6-week-old) soil-grown plants. Col-0, *tt4-11*, and *tt7-5* plants were entrained to a 12 h/12 h LD cycle and moved to continuous light on the first day of data collection. Plants were imaged starting at Circadian Time (CT) 6 every 4 h for 48 h. The hyperspectral imaging system consisted of a Pika L hyperspectral imaging camera (385.6–1027 nm spectral range with 2.0 nm resolution), linear translation stage, mounting tower, lighting assembly, and a desktop computer loaded with Spectronon-Pro software to control the imager and translation stage during imaging (Resonon, Bozeman, MT, USA). This software was also used to measure reflectance values from nine leaves per plant and the average was used to calculate the reflectance values per plant per time point. Statistical analysis of rhythmicity was performed using meta_2d *P*-value in MetaCycle, combining algorithms JTK_CYCLE, ARSER, and Lomb-Scargle (Wu et al., 2015). Spectrophotometric quantification of chlorophyll content was carried out largely as described in Yoo et al. (2019). Briefly, 4.5-week-old plants were transferred to LL and aboveground tissues from individual plants were harvested, weighed, quick-frozen in liquid nitrogen, and stored at −80°C prior to processing. Chlorophyll was extracted in 1.5 ml DMSO at 65°C for 1 h. Absorbance of 50 μl aliquots was determined at 648 and 665 nm using a Cytation 5 plate reader (BioTek, Winooski, VT,

USA) and content of chlorophyll *a* and *b* per μg wet weight was determined using the equations in Barnes et al. (1992).

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AUTHOR CONTRIBUTIONS

BSJW and SK conceived the project. SBH, ESL, LCC, GCP, and BSJW performed the experiments and statistical analyses. BSJW wrote the manuscript with input from the other authors.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Period and phase of *CCA1p::LUC* and *TOC1p::LUC* expression in *tt4-11* and *tt7-5* seedlings relative to the wild-type parental line.

Figure S2. Hyperspectral imaging at 469 nm of leaves from mature plants over a 48-h time course.

Table S1. Primers used for qPCR.

Dataset S1. Full RNA-Seq data with annotations.

Dataset S2. High-confidence differentially expressed genes (DEGs) ($|\log_2(\text{fold change})| \geq 1.0$) and comparisons with previously published lists of: (1) circadian-regulated genes in Arabidopsis (Covington et al., 2008); (2) genes with proximal *CCA1* binding sites (Nagel et al., 2015); (3) genes encompassing the universal stress transcriptome (Ma & Bohnert, 2007); and (4) genes represented in reactive oxygen species (ROS)-responsive transcriptomes (Gadjev et al., 2006).

Dataset S3. DEGs with $|\log_2(\text{fold change})| \geq 0.50$ and $P < 0.05$.

Dataset S4. Panther overrepresentation analysis.

Dataset S5. Comparison of RNA-Seq and previously-published microarray results.

OPEN DATA BADGE

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196636.

DATA AVAILABILITY STATEMENT

The transcriptomics data described in this study have been deposited at the NCBI Gene Expression Omnibus (GEO) archive at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196636.

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