SHORT COMMUNICATION

e-Xtra*

Rhizosphere Microbes Influence Host Circadian Clock Function

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ABSTRACT

The circadian clock is an important determinant of fitness that is entrained by local conditions. Aside from abiotic factors, individual pathogenic soil bacteria affect circadian clock function in plant hosts. Yet, in nature, plants interact with diverse microbial communities, and the effect of complex communities on clock function remains unclear. In *Arabidopsis thaliana* and its wild relative, *Boechera stricta*, we used diverse rhizosphere inoculates and host genotypes to test the effect of complex rhizosphere microbial communities on the host circadian clock. *A. thaliana* plants with an intact rhizosphere microbiome expressed a circadian period closer to 24 h in duration and significantly shorter (by 48 min on average) relative to plants grown with a disrupted

microbiome. Wild-type host genotypes of *A. thaliana* differed in clock sensitivity to microbes, with one genotype (Landsberg *erecta*) expressing a 119-min difference in circadian period length across rhizosphere microbial treatments. A similar pattern of clock sensitivity to soil microbes was observed in *B. stricta*. Finally, rhizosphere microbes collected from the mutant genotype *toc1-21* of *A. thaliana* with a short-period phenotype and used as inoculate significantly shortened the long-period phenotype of the clock mutant genotype *ztl-1*. The results indicate that complex rhizosphere microbial communities affect host clock function.

Keywords: ecology, microbiome, rhizosphere and phyllosphere

Across all domains of life, organisms have evolved endogenous timekeeping mechanisms known as circadian clocks (McClung 2006). In plants, the clock regulates a large percentage of the transcriptome (Covington et al. 2008), the metabolome (Greenham and McClung 2015), leaf-level and organismal physiology (Yarkunova et al. 2016), fitness (Rubin et al. 2017), and even the composition of beneficial rhizosphere microbial communities (Hubbard et al. 2018). There are several well-described abiotic factors that synchronize (or entrain) the clock to local environmental conditions, including photoperiod and temperature (Millar 2003a, b; Eckardt 2005; Webb et al. 2019). Furthermore, a recent study has shown that soil microorganisms may be another environmental factor that affect clock function (Zhang et al. 2013). Specifically, Zhang et al. (2013) found that *Arabidopsis thaliana* plants infected with *Pseudomonas syringae* had significantly shorter periods—that is, the duration of

one circadian cycle—than uninfected plants. Zhang et al. (2013) demonstrated that individual pathogenic microbial taxa can affect clock function. In nature, plants interact with complex microbial communities consisting of hundreds to thousands of taxa with varying effects on plant performance (Bulgarelli et al. 2013). Thus, the question remains as to how natural, complex microbial communities may influence clock function.

Microbiomes affect many aspects of host performance (Berendsen et al. 2012; Bulgarelli et al. 2013; Hubbard et al. 2019). Although an organism's own genotype has been considered to be the primary determinant of phenotype for many traits, there is growing evidence that host-associated microbial communities and their genomic functions also contribute to host phenotypes. In some cases, the phenotypic effects of microbial associates exceed the effects of host genetics (Berendsen et al. 2012; Bulgarelli et al. 2013). For instance, a recent study in the *Arabidopsis* relative *Boechera stricta* has shown that the rhizosphere microbiome can have a stronger effect than genotype on plant response to insect herbivores (Hubbard et al. 2019). The genetic pathways underlying clock function are well described in plants (Millar et al. 1992), yet the magnitude of microbial effects on clock phenotypes is unknown. Comparing the effects of diverse inoculation treatments and host genotypes would reveal relative microbial versus host genetic contributions to clock phenotypes.

In the current study, we tested the effects of complex rhizosphere microbial communities on host clock function. We first characterized the effects of rhizosphere microbes on the host plant clock by

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comparing the circadian period of plants grown in intact versus disrupted rhizosphere microbiome treatments in both the model plant *A. thaliana* and its wild relative, *B. stricta*. Next, we examined the relative effects of distinct microbial treatments versus plant genotypes on circadian period. We then tested whether rhizosphere microbial communities found in association with host plants with short (20 h) versus wild-type (24 h) circadian periods can rescue (shorten) the long-period phenotype (28 h) of a mutant genotype with disrupted clock function.

MATERIALS AND METHODS

Plant material and growth conditions. To investigate the influence of rhizosphere microbes on host plant circadian period, we used *A. thaliana* accessions that harbor the reporter gene *LUCIFERASE* (*LUC*), the gene responsible for bioluminescence in the presence of the substrate luciferin in fireflies (*Photinus phyllis*), linked to the native circadian gene, *COLD CIRCADIAN RHYTHM RNA BINDING 2* (*CCR2*), allowing for quantification of circadian parameters (Millar et al. 1992). Genotypes included in the current study were Columbia (Col), Strand, Wassilewskija, Landsberg *erecta* (Ler), and both the short-period mutant *TIMING OF CAB EXPRESSION 1* (*toc1-21*) and the long-period mutant of *ZEITLUPE* (*ztl-1*) in the Col background.

Seed were surfaced sterilized using a 10% bleach and 0.1% Tween solution, planted into 96-well plates containing sterilized Murashige and Skoog mineral plant growth media containing sucrose at 30 g/liter, and cold stratified for 7 days at 4°C. To germinate and entrain plants, plates of planted seed were placed in a Percival PGC-9/2 growth chamber (Percival Scientific, Perry, IA, U.S.A.) set on a cycle of 12 h of light and 12 h of darkness at 22°C. After 7 days, plants were inoculated with 10 µl of a microbial inoculant created by combining 10 g of soil with 90 ml of reverse-osmosis H₂O and filtered through 1,000-, 500-, and 212-µm sieves to remove nematodes that could detrimentally affect plant performance (van de Voorde et al. 2012). At 24 h after inoculation, plates were moved into an ORCA-II ER digital camera (Hamamatsu Photonics, Bridgeport, NJ, U.S.A.) for imaging and quantification of bioluminescence.

Each 96-well plate contained three genotypes in four rhizosphere microbiome treatments with eight replicates. Rhizosphere microbiome treatments used in this study included intact, disrupted, Col, and *toc1-21*. To generate the intact treatment, we used bulk soil from the Catsburg site in North Carolina that has a well-documented occurrence of wild *A. thaliana* plants (Mauricio and Rausher 1997). For the disrupted treatment, intact inoculant was autoclaved or filtered through 0.2-µm mesh to disrupt microbial communities while maintaining the nutrient load found in the intact treatment. We did not observe significant differences in period when autoclaving versus filtering for the disrupted control and, thus, pooled the samples into the disrupted treatment. The Col and *toc1-21* rhizosphere microbiome treatments reflect rhizosphere microbial communities collected from the Col and *toc1-21* *A. thaliana* genotypes that had been initially inoculated with the Catsburg inoculant and were grown in the same growing conditions as described above. The Col and *toc1-21* rhizosphere treatments were included to determine whether plant-associated microbial communities might differ in their effects on clock period and, specifically, whether microbial communities found in association with a short-period mutant versus wild-type host might differentially affect (shorten) the circadian period of the long-period mutant, *ztl-1*, which typically expresses a circadian period of approximately 28 h. All experiments were replicated two to three times. The inoculums used here were derived directly from soils used in two previous studies, and are known to have significant compositional differences in their rhizosphere

communities and effects on host plant performance (Hubbard et al. 2018, 2019).

Circadian imaging. Prior to imaging plants, 20 µl of 100 mM D-luciferin monopotassium salt and 0.01% Triton X-100 solution was added to each well. Exposure images (30 min long) were taken every hour for 160 h to quantify bioluminescence. Traces were then trimmed to 120-h windows for all samples in all treatments, and visually inspected to ensure quality (Millar et al. 1995). Traces that failed quality control for reasons such as lack of bioluminescence or a failure to show rhythmicity were discarded. High-quality traces were then uploaded to Biodare2 (<https://biodare2.ed.ac.uk>) to estimate circadian period and phase using FFT-NLLS (fast Fourier transform nonlinear least squares) for parameter estimation (Zielinski et al. 2014) (Supplementary Fig. S1). We used two-way analyses of variance (ANOVAs) to characterize the effects of rhizosphere microbiome treatment, plant genotype, and their interaction on circadian period and phase, and Tukey's honestly significant difference post hoc contrasts to characterize differences between genotypes and rhizosphere microbiome treatments. Treatment was often significant for circadian period, whereas results for phase failed to meet the significance threshold, potentially due to greater measurement and estimation error relative to period, and are not discussed further.

Replication in the wild *A. thaliana* relative *B. stricta*. To further characterize the effects of the rhizosphere microbiome on plant clock function, we grew 15 replicates of two *B. stricta* genotypes with natural variation in clock period—genotype 15 (approximately 22-h period) and genotype 14 (approximately 23-h period)—in intact and disrupted soil microbiome treatments. This experiment differed from the *A. thaliana* experiment in three ways. First, we used a leaf movement assay as described by Salmela et al. (2016) in place of the bioluminescence assay, because these *B. stricta* genotypes did not contain the *CCR2:LUC* reporter construct; measurement windows (120 h) and trace analyses for the leaf movement data were otherwise the same as those applied to data from *A. thaliana*. Next, rather than using growth media, plants were grown in sterilized potting mix and were then assigned to either intact versus disrupted soil microbiome treatments; the inoculums were created using soil from the genotypes' home site (South Brush Creek, WY, U.S.A., 41.331°N, -106.504°W) described by Hubbard et al. (2018), with the intact versus disrupted inoculums prepared as for *A. thaliana*. Finally, for this experiment, entrainment conditions simulated local fall conditions experienced by this species, as in Salmela et al. (2016). As for *A. thaliana*, two-way ANOVAs were used to characterize the effects of rhizosphere microbiome treatment, plant genotype, and their interaction on circadian period.

RESULTS

Effects of intact versus disrupted microbiome treatments on clock function. Rhizosphere microbiome treatment strongly affected host circadian period. In *A. thaliana*, there were significant differences in circadian period between genotypes grown in intact versus disrupted microbiome treatments (Fig. 1) ($P < 0.001$), where the circadian period was 48 min shorter on average and closer to 24 h among plants grown with an intact microbiome relative to plants grown with a disrupted microbiome. Likewise, the circadian period of *B. stricta* genotype 14 was significantly shorter when grown in the intact versus disrupted treatment (Fig. 2) ($P = 0.032$).

Genotype-microbiome effects on clock function. For *A. thaliana*, there were significant interactions between rhizosphere microbiome treatment and plant genotype ($P < 0.001$), where all genotypes other than Col were significantly affected by microbiome treatment (Fig. 1). Ler was most responsive to soil microbiome treatment, because replicates grown in the disrupted treatment had a

119-min longer period than plants grown in the intact treatment. Similarly, for *B. stricta*, genotype 15 was not significantly affected by microbiome treatment but there was a >30-min difference in period across treatments for genotype 14, where replicate plants grown in

Fig. 1. Microbiome treatment, intact (blue) versus disrupted (orange), significantly alters plant circadian period ($P < 0.001$), as estimated by bioluminescence quantification. Similarly, plant genotype ($P < 0.001$) and interactions between rhizosphere microbiome treatment and plant genotype ($P = 0.002$) significantly affect plant circadian period. Gray points and bars represent median and standard error, respectively. Genotypes: Col = Columbia, Ler = Landsberg *erecta*, Nrwl = Strand, Ws = Wassilewskija, and *ztl-1* = mutant *ZEITLUPE*. Asterisks denote significant differences between intact versus two disrupted rhizosphere microbiome treatments within a given genotype: * and ** indicate $P < 0.05$ and 0.01 , respectively, and ns = nonsignificant.

Fig. 2. Microbiome treatment affects circadian period in *Boechera stricta*. Replicates of genotype 14 grown in the intact (blue) versus disrupted (orange) differ significantly in circadian period ($P = 0.032$), as estimated by leaf movement assay. Gray points and bars represent median and standard error, respectively. Asterisks denote significant differences between intact versus two disrupted rhizosphere microbiome treatments within each genotype: * indicates $P < 0.05$ and ns = nonsignificant.

the intact microbiome treatment had a significantly shorter period duration than plants grown in the disrupted microbiome treatment (Fig. 2).

Microbial rescue of host clock function. Circadian period of the clock mutant genotype *ztl-1* was significantly shortened when grown with a soil microbiome shaped by the *toc1-21* genotype compared with the microbiome shaped by the Col genotype (Fig. 3) ($P = 0.015$) and also showed a marginally significant shortening when grown with the microbiome of the *toc1-21* genotype versus a disrupted microbiome ($P = 0.101$). Specifically, *ztl-1*'s period was shortened by approximately 150 min when grown in association with the *toc1-21* microbiome relative to the Col and disrupted microbiomes.

DISCUSSION

The rhizosphere microbiome is often referred to as an extension of the plant genome, because complex microbial communities affect many aspects of plant performance (Berendsen et al. 2012; Bulgarelli et al. 2013; Hubbard et al. 2018). However, the extent to which complex rhizosphere microbial communities influence clock function remains unclear. Here, we characterized the influence of the rhizosphere microbiome on host clock function. We specifically addressed the following questions. (i) Do complex rhizosphere microbial communities influence host circadian period? (ii) What are the relative effects of the rhizosphere microbiome versus plant genotype on circadian period? (iii) Can rhizosphere communities shaped by plants with short (21 h) or wild-type (24 h) circadian periods rescue long-period mutant phenotypes?

Fig. 3. Rhizosphere microbiome treatment affects clock period in mutant *ZEITLUPE* (*ztl-1*). The circadian period of *ztl-1* replicates was significantly shortened when grown with rhizosphere microbial inoculate shaped by plants with short periods (21 h, mutant *TIMING OF CAB EXPRESSION 1* [*toc1-21*]) compared with *ztl-1* replicates grown with inoculate shaped by plants with a wild-type period (Columbia [Col]) ($P = 0.015$), as estimated by bioluminescence quantification. The effect of inoculate shaped by *toc1-21* genotypes on circadian period of *ztl-1* was marginally significant compared with the disrupted microbiome ($P = 0.101$). Gray points and bars represent median and standard error, respectively. Letters denote significant differences based on Tukey's honestly significant difference post hoc comparisons between rhizosphere microbiome treatments.

Recent evidence suggests that infection with a single pathogenic microbial taxon, *Pseudomonas syringae*, influences plant clock function through an effect on immune signaling (Zhang et al. 2013). We find that complex communities of rhizosphere microbes significantly affect host plant circadian period (Figs. 1 and 2). It is worth noting that similar results were obtained using both *A. thaliana* (grown in agar media and phenotyped using a reporter gene construct) and *B. stricta* (grown in sterilized soil media and phenotyped using a leaf movement assay); as such, the observation of an accelerated clock in the presence of an intact rhizosphere microbiome is unlikely to derive from artifactual effects of either the microbiome on availability or turnover of luciferin (because luciferin was not used in the *B. stricta* experiment) or of agar media on microbiome composition (because the *B. stricta* plants were grown either in potting mix re-inoculated with local microbes or in noninoculated potting mix and there are known differences in microbial community composition between re-inoculated and noninoculated treatments) (Hubbard et al. 2019). Further research is required to elucidate the mechanisms by which microbes affect plant clock function. However, in an earlier study characterizing the effects of the intact versus disrupted microbial communities used in this study on the plant metabolome, several microbes associated with the intact treatment were known to improve plant access to limiting nutrients such as nitrogen, which can affect clock function and may explain the observations in this study (Haydon et al. 2015; Hubbard et al. 2019).

Regardless of the exact mechanisms by which microbes affect the host clock, the almost 2-h difference in circadian period observed for at least some genotypes (Ler) across the intact versus disrupted rhizosphere microbiome treatments reflects a substantial portion of the range in circadian period expressed by the four wild-type *A. thaliana* accessions used in this study (66%) and by a larger panel of 150 European accessions (33%) (Michael et al. 2003). Thus, rhizosphere microbes play an important role in clock function but microbial effects appear to be of lesser magnitude than host genetic differences. Genotype–rhizosphere microbe interactions suggest that there are segregating host alleles that permit microbes to affect the clock. A larger survey of diverse naturally occurring microbial communities and host genotypes will provide additional insights into the effect of microbial associates on host clock function.

The largest observed changes in clock period could well affect plant fitness, because the mismatch between endogenous and environmental cycle duration can significantly affect performance (de Montaigu et al. 2015; Dodd et al. 2005; Rubin et al. 2017; Salmela et al. 2016). In particular, variation in clock period of similar magnitude to that observed here and expressed in near-isogenic lines affected plant performance in the field (Rubin et al. 2018). Microbial associations with eukaryotes are, of course, evolutionarily ancient and, on average, appear to modulate endogenous host circadian cycles in the current study, although some host genotypes were more sensitive than others to the presence of microbes.

Compositional differences among intact rhizosphere microbiome treatments differentially affected the circadian period of the long-period clock mutant *ztl-1* (Fig. 3). Although rhizosphere microbiomes shaped by the *toc1-21* genotype did not fully rescue clock misfunction in *ztl-1* (i.e., did not restore a 24-h circadian period), the findings presented here suggest that rhizosphere legacy effects could affect circadian period as well as fitness across generations (Rubin et al. 2017).

In sum, we observe that complex microbial communities significantly influence clock function in host plants. Specifically, the effect of the rhizosphere microbiome on circadian period length rivals or exceeds that of differences among some plant genotypes and is equivalent to moderate changes in well-described abiotic inputs, such as a 2-h shift in photoperiod or a change of several degrees in temperature (Gould et al. 2006; McClung 2006). Furthermore, the effect of

rhizosphere microbiomes on circadian clock period is likely sufficient to alter plant fitness, based on prior studies testing the effects of quantitative clock variation on plant performance (Rubin et al. 2017, 2018). Given the pronounced effects that intact microbes have on the plant clock, future work should attempt to identify the specific taxa and mechanisms by which microbes influence the circadian clock.

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