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ORIGINAL ARTICLE

The plant circadian clock influences rhizosphere community structure and function

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Plants alter chemical and physical properties of soil, and thereby influence rhizosphere microbial community structure. The structure of microbial communities may in turn affect plant performance. Yet, outside of simple systems with pairwise interacting partners, the plant genetic pathways that influence microbial community structure remain largely unknown, as are the performance feedbacks of microbial communities selected by the host plant genotype. We investigated the role of the plant circadian clock in shaping rhizosphere community structure and function. We performed 16S ribosomal RNA gene sequencing to characterize rhizosphere bacterial communities of *Arabidopsis thaliana* between day and night time points, and tested for differences in community structure between wild-type (Ws) vs clock mutant (*toc1-21*, *ztl-30*) genotypes. We then characterized microbial community function, by growing wild-type plants in soils with an overstory history of Ws, *toc1-21* or *ztl-30* and measuring plant performance. We observed that rhizosphere community structure varied between day and night time points, and clock misfunction significantly altered rhizosphere communities. Finally, wild-type plants germinated earlier and were larger when inoculated with soils having an overstory history of wild-type in comparison with clock mutant genotypes. Our findings suggest the circadian clock of the plant host influences rhizosphere community structure and function.
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Introduction

In comparison with unvegetated soils, the presence of plants markedly affects the structure of soil microbial communities. Plant roots affect the physical, as well as chemical environment through the exudation of carbon into the rhizosphere zone, which immediately surrounds the roots (Bais *et al.*, 2006; Jones *et al.*, 2009; Dennis *et al.*, 2010). Rhizosphere microbial community structure is dynamic and changes over the course of plant development (Lundberg *et al.*, 2012), in part because of changes in exudation (Chaparro *et al.*, 2014). Although much is known about rhizosphere assembly dynamics on longer time scales, there is currently little information regarding assembly dynamics on shorter, diurnal time scales. Further, although plant exudation may 'feed-down' and affect microbial community structure, rhizosphere communities can 'feed-up' and affect plant performance, by increasing plant access to nutrients (Çakmakçı *et al.*, 2001; Chen *et al.*, 2002; Richardson *et al.*, 2009; Richardson and Simpson, 2011), relieving abiotic stress (Zolla *et al.*, 2013), suppressing pathogens (Mendes *et al.*, 2011, 2013), altering phenology (Wagner *et al.*, 2014; Panke-Buisse *et al.*, 2014) and promoting plant growth (Bashan, 1998; Lugtenberg and Kamilova, 2009; Henning *et al.*, 2016). Some plant species, such as many legumes, have developmental genetic mechanisms that attract explicitly beneficial nitrogen-fixing rhizobia taxa (Bravo *et al.*, 2016). The extent to which plants may attract complex beneficial communities remains largely unclear.

The use of experimental genetic lines available in plant model species may reveal specific genetic paths that affect microbial community structure. Comparing mutant vs wild-type plants of *Arabidopsis thaliana*, Lebeis *et al.* (2015) observed that salicylic acid, an immune signaling molecule,

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altered rhizosphere bacterial community structure. This finding suggests that genes regulating physiological traits, such as immune response, may have a role in shaping rhizosphere communities. Genes regulating additional physiological traits such as gas exchange and specifically carbon assimilation may also be worth examining, because of their effects on the photoassimilate pool available for allocation to root growth and for carbon exudation into the rhizosphere. More generally, the comparison of phenotypes between single-locus mutant genotypes with those expressed by wild-type genotypes removes potentially confounding effects of variation segregating elsewhere in the genome, and enables isolation of pathway-specific effects. Naturally occurring large-effect alleles at causal loci could in some cases have a role in shaping microbial community structure in natural plant populations or could be manipulated in crop species to improve plant growth.

Changes in the presence–absence (or abundance) of just a few microbial taxa can affect plant performance, because of the vast number of root-associated microbial cells and functions (Henning *et al.*, 2016). Across diverse ecosystems, community structure and function are related (Tilman *et al.*, 1997; Talbot *et al.*, 2014), including in plant–rhizosphere associations and in cases where microbial community membership changes by one to few taxa. For instance, Henning *et al.* (2016) observed that the addition of single bacterial taxa to the rhizosphere of *Populus trichocarpa* led to drastic changes in plant growth traits. Similarly, Zolla *et al.* (2013) observed differences in drought response by *Arabidopsis thaliana* plants grown in soil that differed in community structure as a consequence of overstory history. Thus, differences in rhizosphere community structure can lead to differences in rhizosphere community function as estimated from plant performance.

In the current study, we tested the role of the plant circadian clock in determining rhizosphere community structure and function, where function was measured as plant performance. The circadian clock regulates up to 30% of the transcriptome, and affects diverse processes including patterned fluxes of carbon into (stomatal conductance, carbon assimilation) and out of (exudation) the plant on a diurnal scale (Watt and Evans, 1999; Harmer *et al.*, 2000; McClung, 2006; Covington *et al.*, 2008; Badri and Vivanco, 2009; Harmer, 2009; Greenham and McClung, 2015). We hypothesized that the circadian clock could shape rhizosphere community structure on a diurnal scale, if community structure responds to diurnally patterned fluxes of carbon into the rhizosphere, that is, we anticipated that microbial community structure might vary over the course of 24 h. We further hypothesized that rhizosphere communities of plant genotypes harboring clock mutations could differ from wild-type plants, because of differences in physiological phenotypes. Specifically, mutations in the clock genes *TIMING OF CAB EXPRESSION 1* and *ZEITLUPE* lead plants to express altered clock period, or the duration of one circadian cycle (Millar *et al.*, 1995; Kim *et al.*, 2005). As a consequence of altered clock function, clock mutants express distinct physiological phenotypes, including reduced carbon assimilation, chlorophyll content and stomatal conductance (and thus root water uptake) relative to wild-type plants under 24 -h environmental cycles (Dodd *et al.*, 2004, 2005). Clock misfunction may influence rhizosphere communities, if for instance the reduced flux of carbon into plants influences the flux of carbon exudation (Thornton *et al.*, 2004) or if shifts in plant water use alter soil water potential and nutrient availability and hence the rhizosphere environment (Matimati *et al.*, 2014). Finally, if rhizosphere community structure is altered by mutations in clock genes, then we hypothesize there may be differences in community function in the form of plant performance, in which microbial communities shaped by wild-type genotypes may lead to improved plant performance in comparison with microbial communities found in association with clock mutant genotypes.

Materials and methods

Plant material and growth conditions

To investigate the role of the circadian clock in shaping rhizosphere community structure and function, we used the *Arabidopsis thaliana* accession, Wassilewskija (Ws, CS2360), and two circadian clock period mutants in the Ws background, *TIMING OF CAB EXPRESSION* 1 (*toc1-21*) and *ZEITLUPE* (*ztl-30*). *toc1-21* is a short-period mutant (~ 20 h), while *ztl-30* is a long-period mutant (~28 h) in free-running conditions (Kevei *et al.*, 2006; Fujiwara *et al.*, 2008). Many prior studies have shown that the resonance between endogenous and environmental cycles affects plant phenotypes and performance (Dodd *et al.*, 2005; Yerushalmi and Green, 2009; de Montaigu *et al.*, 2015; Salmela *et al.*, 2016); the current experiments extend prior research to test effects of the plant host clock on the rhizosphere microbiome.

For each experiment, seeds were surface sterilized using 15% bleach, 0.1% *Tween* and 84.9% RO H_2O solution, cold stratified in the dark in 1 ml of RO H_2O for 5 days at 4 °C, and placed in RO H_2O to germinate in a Percival PGC-9/2 growth chamber (Percival Scientific, Perry, IN, USA) to ensure synchronous germination. Throughout this study, the growth chamber environment was set to 12/12 light–dark cycle (lights came on at 0700 hours and turned off at 1900 hours), 22 °C/18 °C day–night temperature cycles, 40% relative humidity, and photosynthetic photon flux density = 350 μmol photons m^{-2} s^{-1}. Upon the observation of root radicles, seedlings were aseptically transferred to 2 inch diameter pots filled

with a mixture of sterilized potting media (N ~ 400 p.p.m., P ~ 90 p.p.m.) and microbial inoculate. To generate our sterilized media, Redi-Earth Potting Mix (Sungro Horticulture, Agawam, MA, USA) was autoclaved twice for 60 min. Next, 2 ml of microbial inoculate was added to each pot. The microbial inoculate was created by mixing 360 ml of RO H_2O with 40 g of soil from the Catsburg region in Durham, North Carolina, USA (36.062294°N, –78.849644°W) and filtered through 1000 μm, 212 μm, 45 μm sieves, to remove soil nematodes that might negatively impact plant performance (van de Voorde *et al.*, 2012). The Catsburg region has a well-documented history of *A. thaliana* occurrence, which has been naturalized in this region (Mauricio, 1998). Soil from the Catsburg region and our sterilized potting mix was characterized at the Colorado State Soil-Water-Plant Testing Lab (Fort Collins, CO, USA); of greatest relevance to microbial growth, the Catsburg and potting soils had similar pH values (5.4 vs 5.3, respectively). Following germination, seedlings were thinned to one plant per pot, and pots were watered at 0700 hours daily.

Experimental design

Experiment 1: temporal changes in rhizosphere community structure. To determine if rhizosphere bacterial communities are diurnally dynamic, replicates of wild-type Ws plants were grown for 4 weeks as described above. Starting at 0600 hours on 21 July and ending at 0600 hours on 22 July, 10 replicates were selected at random and harvested every 6 h for rhizosphere soil by separating the roots from the rosette (N = 50), removing closely adhering soil particles from the roots as described in Bulgarelli *et al.* (2012), and storing the samples at –80 °C.

Experiment 2: candidate drivers of rhizosphere community structure. To characterize the effects of circadian period misfunction on rhizosphere bacterial community structure, 10 replicates of Ws, *toc1-21* and *ztl-30* genotypes were planted in a fully randomized design and grown for 4 weeks. Rhizosphere samples were collected as described above at 1800 hours on 21 July and stored at –80 °C. All samples were collected before visible signs of bolting, or the transition from a vegetative to a reproductive state, to avoid confounding effects of plant developmental stage (Lundberg *et al.*, 2012; Chaparro *et al.*, 2014). At the end of this experiment, we collected additional rhizosphere soil from four replicates of each of the three genotypes to generate the inoculum for Experiment 3.

Experiment 3: rhizosphere community feedbacks on plant performance. To test if rhizosphere microbiomes assembled by the three plant genotypes had differential effects on plant performance, we synchronously germinated seeds of the Ws genotype and planted these seeds in sterilized soil media inoculated with soil slurry generated by the Ws, *toc1-21*, or *ztl-30* genotypes and collected at the end of Experiment 2 (N = 60; 20 replicates × 3 inoculates). To determine the effects of the rhizosphere microbiome treatment on plant performance, rosette diameter was measured weekly for 3 weeks. In a second experiment, we allowed seeds to germinate naturally in sterilized soil media inoculated with the same soil slurries (N = 60; 20 replicates x 3 inoculates). For this experiment, seeds were checked daily for germination as estimated from the first observation of cotyledons.

DNA extraction and amplicon sequencing

To extract microbial DNA, rhizosphere samples were placed into 15 ml Nunc Conical Centrifuge Tubes (Thermo Scientific, Waltham, MA, USA) containing 3 ml of phosphate-buffered saline, and then agitated for 15 min to separate soil particles from plant roots as described in Bulgarelli *et al.* (2012). Plant roots were then removed with sterilized forceps and the samples were centrifuged for 15 min at 3000 rcf. The supernatant was discarded, and 0.25 g of the pellet was put into bead tubes from the Mobio Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) using sterilized disposable spatulas. DNA was extracted from each sample following the manufacturer's instructions. With each round of extractions, a soilless blank was included as a negative control. At the end of each round, PCR was performed to ensure sufficient DNA yields and reagent sterility.

DNA extracts were sent to the Marine Biological Laboratories (Woods Hole, MA, USA) for amplicon library preparation of the V4V5 region of the 16S ribosomal RNA gene using the 518 F and 926 R primers (Huse *et al.*, 2014). Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) as described in Nelson *et al.* (2014). Sequence reads were demultiplexed and quality filtered (Phred score ⩾ 20, chimera removal by ChimeraSlayer) using QIIME 1.9.1, uclust was used to perform open reference operational taxonomic unit (OTU) picking at 97% sequence similarity using the Greengenes database (ver. 13.8), and all singletons were removed to avoid the possibility that a sequencing error was called as an OTU (Caporaso *et al.*, 2010; Edgar, 2010; Haas *et al.*, 2011; Bokulich *et al.*, 2012; McDonald *et al.*, 2012). We rarefied to 100 000 reads per sample to ensure common sampling effort. All sequences have been deposited into the Short Read Archive (SRA) under PRJNA391346.

Sequencing data analyses

To describe rhizosphere community structure, we generated Jaccard (presence–absence analysis) and Bray–Curtis (abundance analysis) dissimilarity matrices, and Shannon diversity estimates in QIIME

(Caporaso *et al*., 2010). For Experiment 1, we used adonis to determine if rhizosphere community structure differed between day (1800 hours) and night (0600 hours) time points. To test if shifts in community structure were consistent between day and night time points, we used Pearson's correlation coefficients to compare the percent change in OTU abundance between 0600 and 1800 hours on 21 July and the percent change in OTU abundance between 1800 hours on 21 July and 0600 hours on 22 July. We included only OTUs with >100 reads per sample to avoid potentially confounding effects of low-abundance taxa. For Experiments 2 and 3, we used one-way analysis of variance and Tukey's honest significant differences *post hoc* comparison test using the *car* and *agricolae* R packages to characterize differences in principal coordinates between genotype along axis 1 and to determine differences in plant performance between soil treatments (Fox and Weisberg, 2011; de Mendiburu, 2016). Moreover, OTUs were split into common (> 500 reads) or rare (< 500 reads) categories, and presence–absence analyses and abundance analyses were performed again on the split data sets to determine if effects of clock genotype were detected using common or rare microbial taxa alone. Finally, sequence data were reanalyzed without rarefaction using the R package *Phyloseq*, to determine if results were consistent in the absence of rarefaction (McMurdie *et al*., 2014). Results were similar regardless of rarefaction, that is, the effect of host plant genotype was significant for both binary Jaccard ($P = 0.001$) and Bray–Curtis dissimilarity ($P = 0.001$) analyses with and without rarefaction; here, we present the results of analyses based on rarefaction. All plots were generated using the R package *ggplot2* (Wickham, 2009).

To identify OTUs that explain observed differences in plant performance arising from soil overstory history in Experiment 3, we used the *indicspecies* package for indicator value analysis in R 3.0.3 and *LefSe* on the galaxy web platform (Dufrêne and Legendre, 1997; De Cáceres and Legendre, 2009; Segata *et al*., 2011; R Core Team, 2013). Indicator value analysis (IVA) has been used commonly in ecological studies to ascertain species that underlie treatment or site differences (Dufrêne and Legendre, 1997), and is used here to test which OTU(s) is(are) specific to a given level of a factor (for example, present/abundant in the rhizospheres of Ws replicates and absent from *toc1-21* and *ztl-30* rhizospheres). Notably, the calculation of IVA weights presence–absence and abundance, and as such may be sensitive to rare taxa. *LefSe* performs linear discriminant analysis on sequence data to identify marker taxa that underlie treatment differences, and is weighted preferentially by abundance differences of more common taxa. As rare OTUs contributed to microbiome differences between host plant genotypes, we used both IVA and *Lefse*. Finally, we coarsely estimated microbial community size by dividing the quantity of extracted DNA using a Qubit (ThermoFisher Scientific, Waltam, MA, USA) by the mass of soil used for each extraction to determine if microbial community size influenced plant performance in Experiment 3.

Results

Sequencing results

For Experiment 1, after quality filtering, chimera removal, OTU picking, outlier sample filtering and rarefaction to 100 000 reads per sample (Supplementary Figure 1a), there was a total of 3 700 000 high-quality reads out of 10 250 881 raw reads. For Experiment 2, after similar processing, but rarefaction to 116 000 reads per sample (Supplementary Figure 1b) there was a total 2 668 000 high-quality reads out of 6 487 790 raw reads. The number of reads after each processing step can be found in Supplementary Tables 1 and 2.

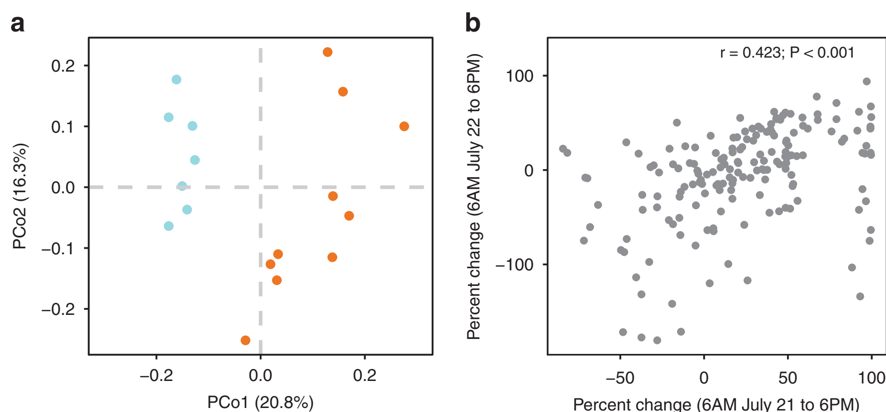

Figure 1 Day vs night timepoint influences rhizosphere community structure. (**a**) Principal coordinate analysis of Bray–Curtis dissimilarities ($n = 17$). Differences between the 1800 hours (day, orange) and 06 00 hours (night, blue) time points were significant at $P = 0.001$. (**b**) Bivariate relationship between percent change in OTU abundance from 0600 to 1800 hours 22 July and percent change in OTU abundance from 0600 hours on 21 July to 1800 hours (r = 0.423; $P < 0.001$).

Experiment 1: temporal changes in rhizosphere community structure

We observed significant differences in rhizosphere communities between day and night time points ($P=0.001$; Figure 1a; Supplementary Table 3). The percent change in OTU abundance between 0600 hours on 21 July and 1800 hours was positively correlated with the percent change in OTU abundance between the 06 00 hours on 22 July and 1800 hours time points ($r=0.423$, $P<0.001$). This relationship suggests that the abundance of many common OTUs shift in a similar manner between day and night time points (Figure 1b).

Experiment 2: candidate drivers of rhizosphere community structure

Rhizosphere community composition (presence vs absence of taxa), abundance, and diversity differed among genotypes (Figure 2). From Jaccard presence–absence analysis, Ws, *toc1-21*, and *ztl-30* rhizosphere communities were significantly different from one another ($P<0.001$; Figure 2a; Supplementary Table 4). PCo1 describes the effect of progressive clock changes between short (lowest PCo values) vs wild-type (intermediate values) vs long (highest values) endogenous period lengths of the plants on rhizosphere community composition. Bray–Curtis relative abundance analysis revealed differences between the rhizosphere communities of the three clock genotypes (Supplementary Table 5), where *toc1-21* communities were different from both Ws ($P=0.02$) and *ztl-30* ($P=0.04$) communities, whereas Ws and *ztl-30* communities were not significantly different from one another ($P=0.39$; Figure 2b). This result suggests that a period length shorter than 24 h specifically alters abundances of OTUs within the rhizosphere community. Similarly, the *toc1-21* rhizosphere communities showed significantly reduced richness and evenness based on Shannon's diversity index in comparison with Ws ($P=0.03$; Figure 2c).

To clarify the contributions of rare vs common OTUs to host plant genotype differences, we analyzed the data when culled to different minimum read numbers (Supplementary Table 6). When culling to a minimum read number > 500 for an OTU (or approximately 1% of the community), Jaccard and Bray–Curtis dissimilarities were significant, indicating that common taxa contribute at least partially to observed differences among the three host plant genotypes in the presence–absence of taxa (Figure 3a) and to differences in OTU abundance between *toc1-21* and both Ws and *ztl-30* (Figure 3b). Communities culled to OTUs with < 500 reads showed significant differences in both composition and abundance, indicating that rare microbial taxa respond to plant genotype (Figures 3c and d). In particular, when data for rare OTUs are analyzed, the distinction between Ws vs *ztl-30* becomes significant ($P=0.001$) (*cf* Figure 2b vs 3d).

Combined, the IVA and *Lefse* analyses identified a total of 13 indicator OTUs associated with the Ws rhizosphere (Figure 4), 12 indicator OTUs associated with the *toc1-21* rhizosphere (Supplementary Tables 7 and 9), and 12 indicator OTUs associated with the *ztl-30* rhizosphere (Supplementary Table 8 and 9). As IVA is more sensitive to rare taxa, the two methods select somewhat different OTUs as biomarkers of host plant genotype. Notably, however, there is significant taxonomic overlap between the OTUs identified by IVA and *Lefse*. That is, taxa identified by IVA are phylogenetically related to those identified by *Lefse*, or *vice versa*. For instance, of the 13 indicator OTUs associated with the Ws rhizosphere, six taxa were members of the phylum Acidobacteria (IVA: DS-100; o_;f_;g_, llb;f_;g_, PAUC26;f_;g_; *Lefse*: Acidobacteria, Solibacterales;f_;g_, iii1_15;f_;g_) and two taxa were members of the Chloroflexi (indicator species analysis: Anaerolineae;o_;f_;g_, *Lefse*: Chloroflexi). From previous studies, some members of both phyla (Acidobacteria and Chloroflexi) and genera (*Agromyces* and *Cellulomonas*), have been described as growth promoting (Egamberdiyeva and Höflich, 2002; Kuffner *et al.*, 2008; Chen *et al.*, 2014; Kielak *et al.*, 2016). Finally, community size as estimated from DNA per unit soil mass ($P=0.11$) and soil pH ($P=0.53$) did not significantly differ across clock genotypes (Supplementary Tables 10 and 11).

Experiment 3: rhizosphere community feedbacks on plant performance

Soil overstory history had a significant influence on early plant performance (Figure 5). Wild-type plants grown in a soil with a history of Ws plants had significantly larger rosette diameters than plants grown in soils with a history of *toc1-21* and *ztl-30* after 1 week (19.4% and 14.4%, respectively; $P=0.002$) and 2 weeks of growth (10.8% and 8.3%, respectively; $P=0.04$). However, at the end of 3 weeks of growth, Ws plants grown in soils conditioned by each of the clock genotypes were only marginally different in size ($P=0.11$). In a germination experiment of similar design (in which seedlings were not transplanted but instead germinated directly on soil), Ws seeds in pots with Ws inoculum germinated an average of 5.2 days earlier than seeds planted into pots with *toc1-21* ($P=0.002$) inoculum and 5.7 days earlier than those planted into *ztl-30* ($P=0.024$) inoculated pots ($P<0.001$; Figure 5b).

Discussion

The rhizosphere microbiome has been referred to as the 'second genome' of plants or the extended phenome (Berendsen *et al.*, 2012). In part, these names reflect the role of the rhizosphere microbiome in determining plant performance. Empirical studies suggest complex feedbacks between plants and

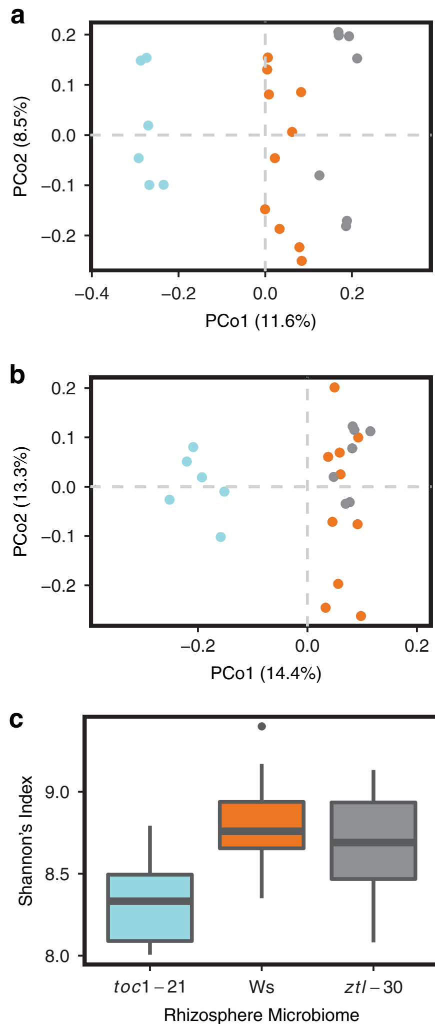

Figure 2 Clock function in *A. thaliana* alters rhizosphere community composition, abundances and diversity. (**a**) Principal coordinate analysis of Jaccard dissimilarities, where rhizosphere communities of Ws are represented by orange circles, *toc1-21:* blue circles, and *ztl-30*: gray circles ($n = 23$). Rhizosphere community composition differs significantly between clock genotypes ($P = 0.001$). (**b**) Principal coordinate analysis of Bray–Curtis dissimilarities ($n = 23$). OTU abundances differ significantly between *toc1-21* and both the Ws and *ztl-30* genotypes ($P = 0.001$). (**c**) Mean Shannon diversity index. The top and bottom of boxes represent the 75th and 25th percentiles, respectively. Whiskers represent 1.5 times the interquartile range. One-way analysis of variance and Tukey's *post hoc* comparisons indicate significant differences between *toc1-21* and both the Ws and *ztl-30* genotypes ($P = 0.03$).

microbes, under which plant species may modulate rhizosphere community structure via carbon exudation and under which microbes may alter plant phenotypes directly or via ecosystem services such as nutrient accessibility (Bulgarelli *et al.*, 2013). The mechanisms by which different plant genotypes may influence rhizosphere community structure remain largely unclear, as are the effects on plant performance of rhizosphere microbiomes selected by the plant host genotype (Heath and Tiffin, 2007; Panke-Buisse *et al.*, 2014; Lebeis *et al.*, 2015). Understanding plant–rhizosphere microbiome interactions is agroecologically relevant because rhizosphere communities can strongly influence plant fitness and biomass, which can in turn inform evolutionary studies of adaptation, conservation, and agronomic practices (Pérez-Jaramillo *et al.*, 2016). In this study, we tested the role of the plant circadian clock as a mediator of plant–rhizosphere microbiome interactions. We hypothesized that (1) rhizosphere community structure may be temporally dynamic, if rhizosphere taxa respond to diurnally patterned fluxes of carbon, water, or nutrient availability into the rhizosphere (or other diurnally patterned plant phenotypes). (2) We further hypothesized that clock misfunction would have a role in shaping community structure, because differences in plant physiology attributable to genotype would lead to differences in rhizosphere community structure. (3) Finally, we hypothesized that differences in rhizosphere community structure attributable to plant genotype could lead to differences in community function with regards to plant performance.

The composition of plant-associated microbiomes is known to shift on long time scales, such as across seasons or across developmental stages of the plant host (Lundberg *et al.*, 2012; Chaparro *et al.*, 2014; Wagner *et al.*, 2016). The short duration of many microbial life cycles means that microbial community composition may also respond to more rapid changes in the environment. Yet, it remains unclear if the community composition of microbes found in association with plants changes on short timeframes, such as across day-night transitions. We observed diurnally patterned shifts in rhizosphere community structure. That is, we observed consistent shifts in rhizosphere communities between day and night time points (Figure 1). Difference in community structure observed between the two time points may reflect the effects of day vs night conditions and carbon, water or nutrient availability in the rhizosphere. Several prior studies have shown that the concentration of certain exudates varies over the course of day (Watt and Evans, 1999; Badri and Vivanco, 2009). For instance, Iijima *et al.* (2003) observed higher rates of mucilage exudation at night, whereas other studies have observed higher prevalence of flavonoids and catechin during day conditions (Hughes *et al.*, 1999; Iijima *et al.*, 2003; Tharayil and Triebwasser, 2010). Further, rhizosphere water is depleted diurnally, depending on root and soil hydraulics (Sperry *et al.*, 1998), and the transpiration stream increases nutrient flow (Matimati *et al.*, 2014), potentially depleting soil nutrients in the rhizosphere zone. Therefore, rhizosphere taxa and populations may vary in abundance depending on soil resource availability, leading to our observed differences in community structure between day and night time points. Future experiments should be designed to tease apart the relative influence of root exudates, water dynamics, and nutrient uptake within the rhizosphere on microbes.

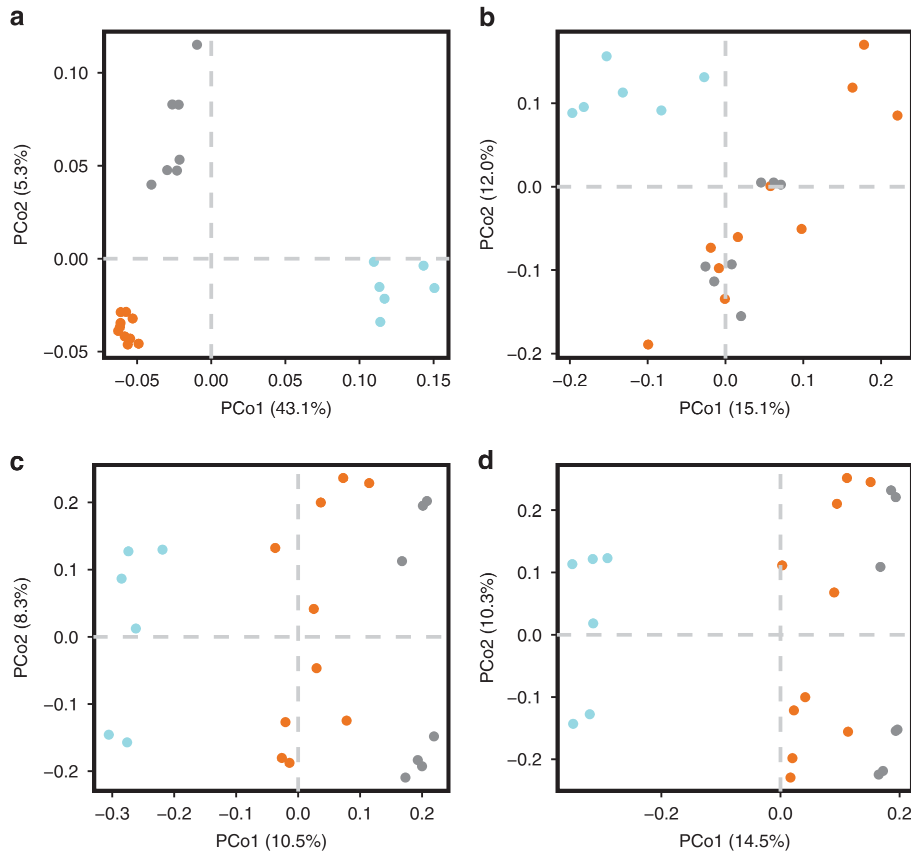

Figure 3 Plant genotype influences both common (> 500 reads) and rare (< 500 reads) rhizosphere taxa. (**a**) Principal coordinate analysis of Jaccard dissimilarities of common taxa, where rhizosphere communities of Ws are represented by orange circles, *toc1-21*: blue circles and *ztl-30*: gray circles ($n=23$). Rhizosphere community composition differs significantly between all clock genotypes ($P=0.001$). (**b**) Principal coordinate analysis of Bray–Curtis dissimilarities of common taxa ($n=23$). OTU abundances differ significantly between *toc1-21* and both the Ws and *ztl-30* genotypes ($P=0.001$). (**c**) Principal coordinate analysis of Jaccard dissimilarities of rare taxa ($n=23$). Rhizosphere community composition differs significantly between all clock genotypes ($P=0.001$). (**d**) Principal coordinate analysis of Bray–Curtis dissimilarities of rare taxa ($n=23$). OTU abundances differ significantly between Ws and both *toc1-21* and *ztl-30* genotypes ($P=0.001$).

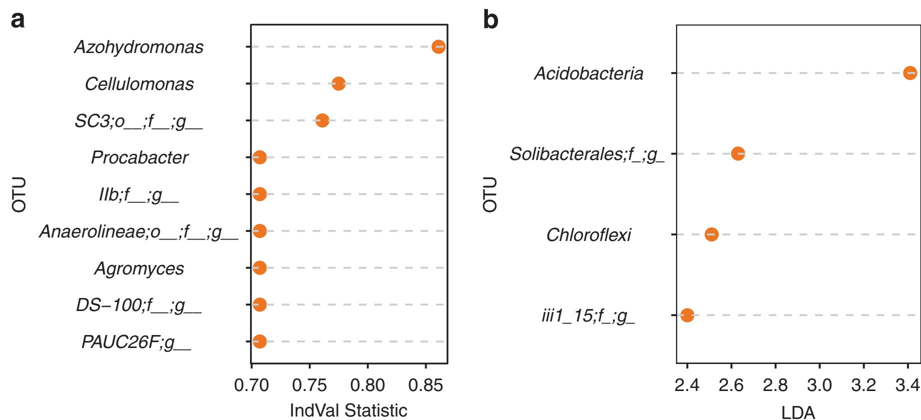

Figure 4 Indicator taxa based on (**a**) IVA and (**b**) *Lefse*.

Specific plant genes have important roles in shaping rhizosphere communities (Bravo *et al.*, 2016), and here we observed that circadian clock genes significantly influence rhizosphere community structure. In the current study, plant genotype explained 19.1% of the variation in community composition (presence vs absence of taxa), 21.7% of the variation in community relative abundances, and brought about differences in community diversity between short (*toc1-21*) vs longer (Ws, *ztl-30*) period genotypes (Figure 2). These differences in communities explained by clock genotype surpass variation explained by genotype in previous studies of the influence of plant genotype on rhizosphere

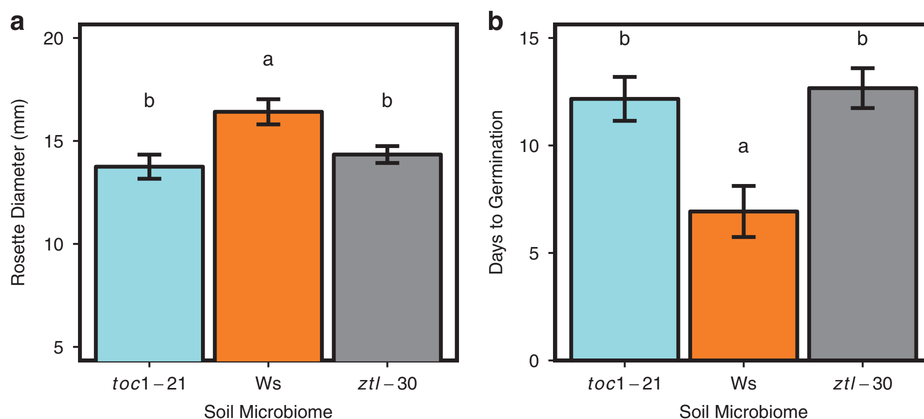

Figure 5 Effects of soil overstory history on plant growth. Letters denote significant differences between soil treatments based on Tukey's Honest Significant Differences *post hoc* comparisons. (**a**) At week 1 ($n = 60$), plants grown in a soil with a history of Ws had significantly larger rosette diameters than plants grown in soils with a history of *toc1-21* or *ztl-30* ($P = 0.002$). (**b**) In a separate experiment where seeds were not germinated synchronously ($n = 35$), seeds sowed onto a soil with a history of Ws occurrence germinated significantly earlier than seeds sown into soils with a history of *toc1-21* or *ztl-30* genotypes ($P < 0.001$).

community structure (Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2012; Peiffer *et al.*, 2013; Lebeis *et al.*, 2015). Our results thus suggest that circadian clock misfunction has a strong influence on rhizosphere community structure. The large percent variance explained may arise from the pervasive transcriptomic and phenotypic effects of clock misfunction on the plant host, and potentially the microbial inoculant used here is one that amplifies the effect of host genotype, as demonstrated in other studies (Weinert *et al.*, 2011; Peiffer *et al.*, 2013).

TIMING OF CAB EXPRESSION 1 had a particularly pronounced impact on rhizosphere community structure, because strong mutant alleles in this gene led to changes in community composition, abundance and diversity (Figure 2). On the other hand, disruption of *ZEITLUPE* had less of an influence on rhizosphere community structure, with its effect limited to differences in OTU presence–absence relative to Ws (Figure 2). One possible explanation for the asymmetric effects of clock misfunction is that long period lengths theoretically enable better phase adjustment to dawn, such that period lengths shorter than 24 h may have more detrimental fitness consequences in nature (or in this case lead to greater deviations in rhizosphere microbial community structure) in comparison with period lengths greater than 24 h (Johnson and Kondo, 1992; McClung, 2006; Kevei *et al.*, 2006; Hotta *et al.*, 2007). Regardless of the exact mechanisms, clock misfunction and the mismatch between endogenous plant cycles and exogenous cycles affected aspects of microbial community structure.

As in any ecosystem, there is a link between community structure and function (Tilman *et al.*, 1997). Several studies have illustrated this relationship in plant–rhizosphere microbiome interactions, where differences in plant performance can be attributed to differences in rhizosphere community structure (Mendes *et al.*, 2011, 2013; Zolla *et al.*, 2013; Wagner *et al.*, 2014). Here, differences in rhizosphere communities (arising from mutations in host clock genes) affected performance of wild-type Ws plants grown in soils with differing overstory histories (Figure 5). Ws plants performed best when exposed to an inoculum from soils in which wild-type rather than clock mutant genotypes had been grown. We observed differences in the timing of germination, where Ws seeds sown in soils with a history of Ws occurrence germinated earlier. In comparison with untreated soil, autoclaved soil, such as that used here, differs in both chemical and physical properties and reflects a novel and possibly more stressful environment for plants (Trevors, 1996; Brulé *et al.*, 2001; Lau and Lennon, 2011); differences in germination observed here may therefore reflect that the microbes from Ws-conditioned soil enable normal germination under the novel autoclaved soil conditions (rather than an acceleration of germination timing under natural conditions *per se*) (Lau and Lennon, 2011; Mahmood *et al.*, 2014). Beyond germination timing, we observed that wild-type plants were also larger when grown in soils with a history of Ws rather than mutant genotype growth. These findings from two experiments in which soils were independently conditioned by Ws vs mutant genotypes suggest first that plants can select explicitly beneficial soil communities that improve initial offspring performance, and second that disruption of these communities by mutations in clock genes adversely affects initial offspring phenology and growth.

Although additional research is required to ascertain causality, community composition patterns and indicator analyses provide hypotheses as to which OTUs may lead to these differences in performance (De Cáceres and Legendre, 2009; DeAngelis *et al.*, 2015). Here, we identified 13 indicator OTUs associated with Ws rhizosphere. Parallel to the differences in microbial community structure among host plant genotypes (Figures 2 and 3), one possibility is that rare OTUs underlie differences in plant performance observed between the Ws and *ztl-30* rhizosphere microbiomes, whereas rare and common

OTUs could contribute to plant performance differences observed between the Ws and *toc1-21* microbiomes. Rare OTUs could affect plant performance via so-called indirect effects, such as facilitation of or competition with explicitly plant growth-promoting microbes, whereas common microbes could promote plant growth through indirect or direct interactions (Saleem *et al.*, 2016). Specifically in regard to the indicator species analyses (IVA and *Lefse*), the presence and abundance of Acidobacteria (Kielak *et al.*, 2016), Chloroflexi (Chen *et al.*, 2014), *Cellulomonas* (Egamberdiyeva and Höflich, 2002) and *Agromyces* (Kuffner *et al.*, 2008) in the Ws rhizosphere may explain the differences in plant size between rhizosphere treatments, as these OTUs have been previously associated with plant growth promotion.

In sum, we have shown that the plant circadian clock shapes rhizosphere community structure, particularly the presence of rare taxa. Further, this plant genetic driver of community assembly also influences community function, as estimated from plant performance. As community structure may shift in response to day and night conditions, future characterizations of the rhizosphere should account for differences in community structure attributable the timing of rhizosphere collection. Finally, additional research is needed into the role of host plant physiological loci in shaping rhizosphere communities.

Conflict of Interest

The authors declare no conflict of interest.

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