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Note

Use of a duckweed species, *Wolffiella hyalina*, for whole-plant observation of physiological behavior at the single-cell level

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Abstract We developed a new model system to analyze physiological behavior at the single-cell level in whole plants. *Wolffiella hyalina* is a species of rootless duckweed, which has a thin and very small structure and can grow rapidly on the surface of culture medium. Epidermal and mesophyll cells were transfected with a reporter gene using particle bombardment and were observed at the single-cell level in the whole living plant. An EM-CCD camera system with a macro zoom microscope was used to capture time-lapse images of bioluminescence, and we successfully detected circadian rhythms in individual cells that expressed a luciferase gene under the control of a circadian promoter. We also detected individual *S*-phase cells in meristematic tissues of intact *W. hyalina* plants by using a 5-ethynyl-2′-deoxyuridine (EdU)-labeling assay. Our observations indicated that low-molecular-weight compounds could access the inside of the plant body. Thus, *W. hyalina* showed the experimental characteristics suitable for single-cell analyses that could be combined with whole-plant observations and/or pharmacological analyses/chemical biology.

Key words: bioluminescence monitoring, circadian rhythm, EdU, single-cell analysis, *Wolffiella hyalina*.

Cyclic processes, such as circadian rhythms, affect many aspects of plant physiology (Sweeney 1987). Circadian rhythms are temporal events controlled by a circadian clock based on a cell-autonomous oscillator that can be synchronized to daily environmental cycles. In addition to environmentally coupled cyclic processes such as circadian rhythms, rhythmic phenomena such as the cell cycle show periodicity that is basically independent of environmental periodicity. Both the circadian rhythm and the cell cycle are cyclic processes occurring at the cell level and they are spatially and temporally coordinated in the plant body. Thus, analysis of physiological behavior at the single-cell level in the whole plant is important for understanding the physiology of these cyclic processes (Libault et al. 2017; Muranaka and Oyama 2018). Circadian rhythms at the single-cell level have been analyzed in intact plants such as the duckweed *Lemna gibba* following transfection with a circadian bioluminescence reporter using the particle bombardment method (Muranaka and Oyama 2016; Muranaka et al. 2013; Okada et al. 2017). These analyses clearly demonstrate heterogeneous circadian behaviors in individual cells in the same frond (leaf-like structure of duckweed). Although circadian rhythms basically occur in every cell of the plant body, cell division occurs at specialized regions known as meristems. Local cell-division parameters, such as the cell division plane, cell division rate, and distribution of dividing cells in the meristem are important in determining the organization of plant tissues. A quantitative analysis of dividing cells in plant tissues can be achieved by the EdU-labeling assay, which identifies *S*-phase cells (Kotogány et al. 2010). EdU is a thymidine analog that is incorporated into DNA during DNA synthesis (Salic and Mitchison 2008). EdU-labeled nuclei/cells are then stained with a fluorescent dye that allows the cells to be identified by microscopy or flow cytometry (Kotogány et al. 2010). This method has been used to investigate cell cycles in a range of plant tissues in young seedlings, roots, and tissues/organs excised from the plant body (Bass et al. 2014). However, it has not been applied to meristematic tissues in the shoots and leaves of intact mature plants due to the limited accessibility of EdU into these tissues.

In the present study, we approached whole-plant observation of physiological behavior at the single-cell level using the duckweed species *Wolffiella hyalina* (strain 8640). *W. hyalina* has a very small and thin plant body and does not have roots (Figure 1; Landolt 1986).

Abbreviations: *CCA1*, *CIRCADIAN CLOCK ASSOCIATED1*; EdU, 5-ethynyl-2′-deoxyuridine.
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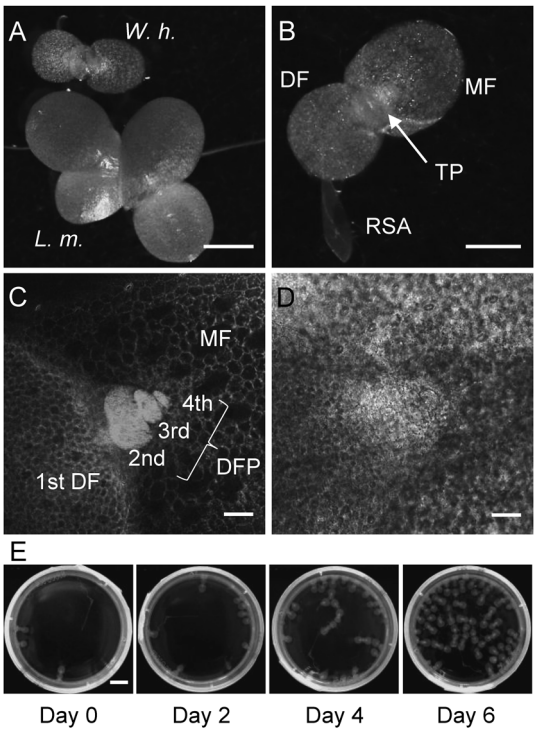

Figure 1. Morphology and growth of *Wolffiella hyalina*. (A) A top view of *W. hyalina* (*W. h.*) and *Lemna minor* (*L. m.*). (B) A bottom view of a *W. hyalina* colony, the mother frond (MF) with a root-shaped appendage (RSA) and a daughter frond (DF). The terminal pouch (TP) of the mother frond is indicated by an arrow. (C) A fluorescent image of a meristematic region. The cell wall was stained with Calcofluor White in a ClearSee-treated colony (Kurihara et al. 2015). Part of the 1st daughter frond (1st DF) and the following daughter frond primordia (DFP) of the mother frond (MF). (D) A bright field image of (C). (E) Growth of *W. hyalina* cultured in a 35-mm dish. A top view image was captured every 2 days. Scale bars: 2 mm in (A), 1 mm in (B), 100 μm in (C), (D), 5 mm in (E).

The plants have only a single terminal pouch (pocket) in which meristematic tissue is present (Figure 1C, D; Landolt 1986). By contrast, *Lemna* plants, which have been frequently used as a model species for physiological experiments, have a root and two lateral pouches (Caux et al. 1988; Prasad et al. 2001). Thus, *W. hyalina* plants have a much simpler body structure. In the present study, we cultured *W. hyalina* plants on modified NF medium with 1% sucrose and 5 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] as previously described for *L. gibba* (Muranaka and Oyama 2016). Plants were maintained under continuous light at 25±1°C, with light supplied by fluorescent lamps (FLR40SEXW/M/36-HG; NEC) at approximately ~50 μE m^{-2} s^{-1}. Under these conditions, the growth rate estimated by the increase in colony number was more than 10 fold per week (Figure 1E), giving a doubling time of less than 2 days.

First, we checked if particle bombardment was applicable to gene introduction into *W. hyalina* colonies as described previously for other duckweed plants (Muranaka et al. 2013, 2015). In brief, we used an 8 μl aliquot of pre-washed gold particle suspension (1 μm diameter) in 50% glycerol (60 mg ml^{-1}) and 1 μl of plasmid DNA (1 μg ml^{-1}). A helium gun device (PDS-1000/He; Bio-Rad) was used for particle bombardment according to the manufacturer's instructions [vacuum, 26 mmHg; helium pressure, 450 psi (rupture disc)]. As a bioluminescent reporter, we used *AtCCA1::LUC* in which a firefly luciferase gene is driven by the *Arabidopsis CCA1* promoter. *AtCCA1::LUC* is a circadian bioluminescent reporter that peaks in the morning and is expressed in almost all cells (Fukuda et al. 2007; Nakamichi et al. 2004). This reporter gene has been shown to work as a circadian marker with a peak around dawn in other duckweed species (Miwa et al. 2006; Muranaka et al. 2015). Single-cell bioluminescence imaging was performed as described previously with a modification of the optical device (Muranaka and Oyama 2016): a macro zoom microscope (MVX-10 with an MVPLAPO 0.63 X lens; Olympus Optical) was used for better spatial resolution. This system can detect luminescent spots scattered across the frond surface (Figure 2A, B). Gene introduction efficiency (luminescent spot density) was comparable with that reported previously for *L. gibba* (Muranaka et al. 2013). We determined the cell types into which the reporter gene had been introduced by particle bombardment. A fluorescent reporter (*CaMV35S::GFP-h*; Nakano et al. 2009) was co-introduced with the bioluminescent reporter into cells. The GFP-h protein localizes at the endoplasmic reticulum, and can be used as a marker for cell shape. Fluorescent cells in the colonies with luminescent spots were examined under a confocal microscope (LSM510-META; Carl Zeiss, http://corporate.zeiss.com/). Two cell types showed fluorescence: cells with a polygonal shape and cells with a round shape (Figure 2C, D). The former showed the characteristic shape of epidermal cells and the latter of mesophyll cells (Landolt 1986). Approximately 80% of transfected cells in the fronds were epidermal cells and the remainder were mesophyll cells (Figure 2E).

Next, we investigated the luminescence behavior of cells into which *AtCCA1::LUC* had been introduced. Circadian rhythms of the plants were entrained (synchronized) by two nights with 12-h dark periods. A single colony of plants located by the wall of the culture dish was anchored on the medium with pins surrounding it (Figure 2A). Single-cell bioluminescence was monitored in this colony under constant light conditions as described previously but with the modification of the optical device (Muranaka and Oyama 2016). To illuminate samples during single-cell monitoring, we used optical fiber-guided white light (30 μE m^{-2} s^{-1}) from a light-emitting diode device (PFB-20SW, CCS Inc.). We analyzed luminescence behavior in cells of a frond in two independent experiments (Figure 2F–H). In both experiments, nine luminous spots on the frond

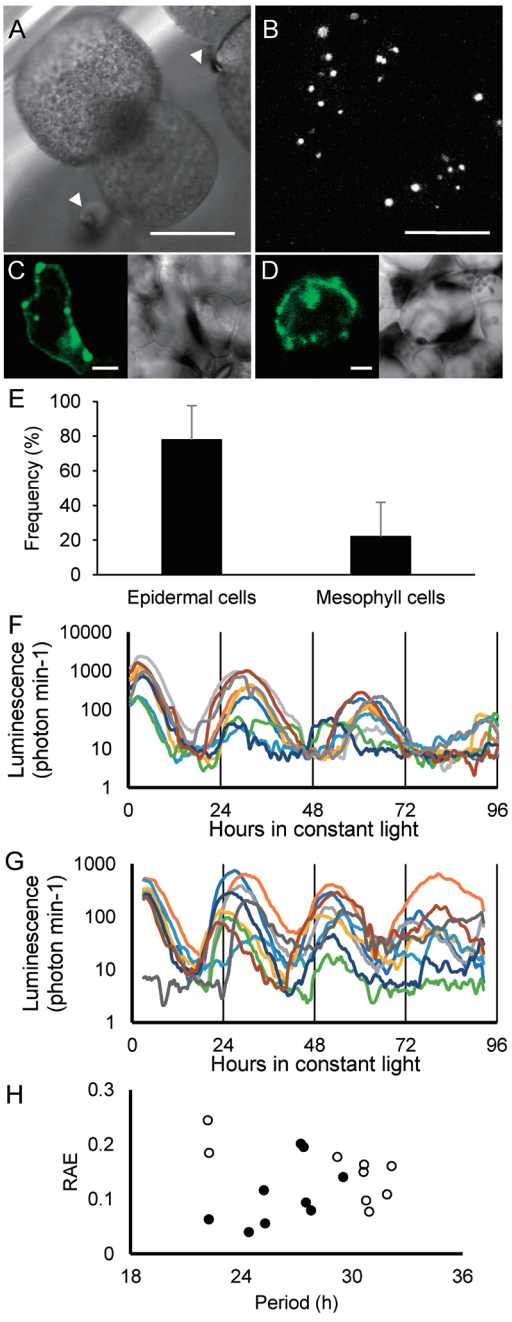

Figure 2. Single-cell analysis of *W. hyalina* cells transfected with reporter genes by particle bombardment. (A, B) A bright field image of *W. hyalina* transfected with the circadian bioluminescence reporter *AtCCA1::LUC* (A), and a luminescence image captured 24 h after particle bombardment (B). The plants were treated with a 12-h dark period that ended 2 h before the capture of the luminescence image. Arrowheads show the positions of pins. (C–E) An epidermal cell (C) and a mesophyll cell (D) that were transfected with the fluorescence reporter gene *CaMV35S::GFP-h*. A fluorescence image (left) and a bright field image (right). The percentages of each type of transfected cells (E). In total, 193 fluorescent cells were observed and classified in three independent experiments. Error bars represent the SD. (F–H) Circadian rhythms of bioluminescence intensities of individual cells. Temporal changes of bioluminescence intensities of nine cells in a frond under constant light are plotted in (F) and (G). The samples differ between (F) and (G). Period lengths and relative amplitude errors (RAEs) of individual cellular rhythms shown in (F) and (G) are plotted in (H). Open- and closed circles represent data from (F) and (G) respectively. Scale bars: 1 mm in (A, B), 20 μm in (C), 10 μm in (D).

maintained luminescence at least for 3 days after the plants were released into the constant light conditions. Every luminous spot showed a circadian rhythm while decreasing its luminescence intensity (Figure 2F, G). In the first experiment, seven cells showed circadian rhythms with relatively high luminescence intensities and their period lengths, as estimated by FFT-NLLS analysis, were ~31 h (29–32 h) (Figure 2F, H). In the FFT-NLLS analysis, the stability of the rhythm is assessed by an index of relative amplitude error (RAE) (Plautz et al. 1997). The other two cells (green and navy-blue lines in Figure 2F) showed dampened rhythms with shorter period lengths (~22 h) (Figure 2H). Their luminescence intensities were much reduced and possibly too low to accurately detect circadian rhythms. In the second experiment, the period lengths of the bioluminescence rhythms of the nine cells varied between 22 and 30 h, with a mean (±SD) of 26.3(±2.1) h (Figure 2G, H). Thus, a relatively large deviation in period lengths of cellular circadian rhythms occurred even in a single frond. The variation between cells resulted in the desynchronization of individual cellular rhythms under constant light conditions (Figure 2G). In the first experiment, such desynchronization was not obvious and individual cellular rhythms with longer period lengths (~31 h) appeared synchronous for at least for 3 days in constant light conditions (Figure 2F). Circadian behaviors of individual cells might vary between fronds as observed in previous studies using *L. gibba* (Muranaka and Oyama 2016). The variation of the period lengths between cells might be due to the difference of cell types because both epidermal- and mesophyll cells were transfected with the circadian reporter gene (Figure 2E).

To analyze cell division, another cyclic process, we used an EdU-labeling assay to detect *S*-phase cells (Kotogány et al. 2010). *W. hyalina* and *Lemna minor* were used as plant materials (Figure 1A). Vigorously-proliferating colonies on NF medium containing 1% sucrose under constant light were incubated for 3 or 12 h (*W. hyalina*), or 24 h (*L. minor*) on the same medium with 20 μM EdU (Invitrogen Click-iT® EdU Imaging Kit) under constant light. The colonies were then fixed for 30 min in 3.7% (w/v) formaldehyde solution in phosphate buffered saline (PBS) with 0.1% Triton X-100. They were passed through a methanol series to remove chlorophyll: 70% (30 min), 80% (30 min), 90% (30 min), and 100% (30 min). They were then washed three times for 10 min in PBS. The colonies were incubated with 200 μl Click-iT® reaction cocktail (Invitrogen Click-iT® EdU Imaging Kit C10637; 176 μl of 1×Click-iT® EdU reaction buffer, 4 μl of copper protectant, 0.48 μl of Alexa Fluor® 488 and 20 μl of 1×Click-iT® EdU buffer additive) for 30 min at room temperature in the dark. The stained colonies were washed once with PBS. EdU-labeled nuclei were identified under an LSM510-META confocal microscope.

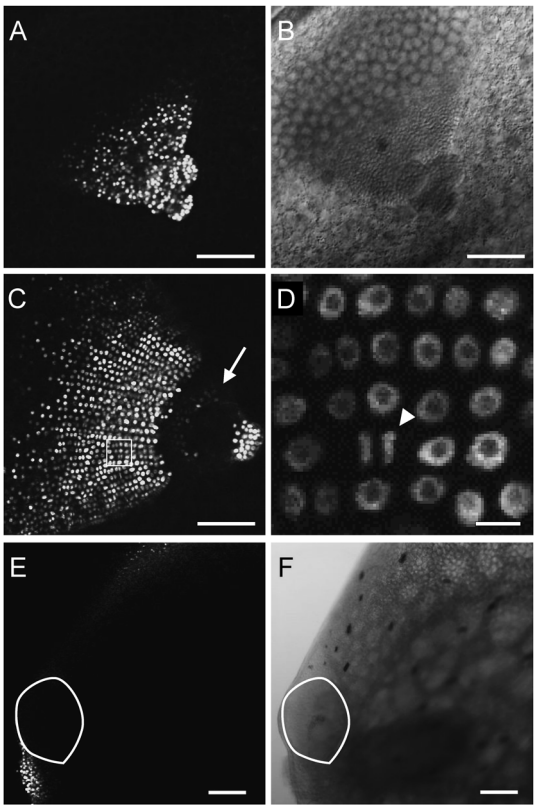

Figure 3. EdU incorporation of meristematic tissues. Colonies of *W. hyalina* (A–D) and *L. minor* (E, F) were incubated for 3 h (A, B), 12 h (C, D), and 24 h (E, F) with 20 μM EdU in the culture medium, and EdU detection was then performed. (A, C) Fluorescence images of a meristematic region. The area without fluorescence signals shown by an arrow in (C) indicates fluorescence signals in another focal plane. (B) A bright field image of (A). (D) A magnified image of the boxed area in (C). The arrowhead shows an EdU-labeled mitotic figure. (E) A fluorescence image of an *L. minor* meristematic region. (F) A bright field image of (E). The white frame areas in (E) and (F) represent the position of the meristematic region (a very young bud). Scale bars: 100 μm in (A–C, E, F), 10 μm in (D).

Fluorescent spots were observed in meristematic tissues in fronds of *W. hyalina* (Figure 3A–D). After 3 h EdU incubation, round fluorescent spots were detected in a sectorial region including several young buds (Figure 3A). After 12 h EdU incubation, the fluorescent spots were detected over a larger sectorial region and appeared aligned in the outer part (Figure 3C). EdU-labeled mitotic figures were found in meristematic tissues in fronds of *W. hyalina* after 12 h EdU incubation (Figure 3D). In contrast to *W. hyalina*, *L. minor* only showed spotted fluorescence signals close to the surface and the cut end of young fronds but not in meristematic tissues even after 24 h EdU incubation (Figure 3E, F). The growth rate of this plant was estimated as 1.4-fold increase per day (Driever et al. 2005); many cells in meristematic tissues performed cell divisions in the period of 24 h. Thus, EdU and/or the reactive fluorescent dye appeared to be inaccessible to young buds inside the frond of *L. minor*. Fronds of *W. hyalina* are much thinner than those of *L. minor*; meristematic tissues in *W. hyalina* are located closely to the lower frond surface from which nutrients in the culture medium are absorbed (Landolt 1986). This structural characteristic of the plant body of *W. hyalina* is likely to cause the good accessibility of the chemical compounds to young frond primordia. Previous studies of the cell cycle used the terrestrial part of the tissues that were excised from the plants for EdU-labeling (Nakayama et al. 2015; Stronghill et al. 2014). By using an intact *W. hyalina* plants as the material for the EdU-labeling assay, the spatial regulation of stages of cell cycle in a whole plant can be investigated. As shown in the labeling assay using EdU (molecular weight (MW)=252.23) and Alexa Fluor® 488 azide (MW=861.04), low-molecular-weight compounds with similar molecular weights appear to be able to access cells inside the *W. hyalina* plants. This characteristic will be a great advantage for other physiological assays using chemical compounds.

W. hyalina is suitable for whole-plant observation because of its very small and thin structure and its rapid growth. Its fronds are flat, immobile in the vertical direction on the surface of medium, making it unnecessary to adjust focus for imaging during long-term monitoring. We succeeded in monitoring bioluminescence reporter gene expression in *W. hyalina* plants at the single-cell level as was previously performed in *L. gibba* (Muranaka et al. 2013). Single-cell analysis of the plant body should lead to a better understanding of various plant physiological behaviors (Muranaka and Oyama 2018). As shown in the EdU-labeling assay, *W. hyalina* plants appear to allow easier access to low-molecular-weight compounds than *Lemna* plants. Thus, *W. hyalina* will be a useful tool not only for cell cycle analysis but also for pharmacological experiments. By combining single-cell analysis on the plant body and pharmacological approaches, including chemical biology, it is expected that the mechanisms of various cyclic phenomena exhibited by the plants will be unraveled.

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