Instrumentation and Software for Analysis of Arabidopsis Circadian Leaf Movement

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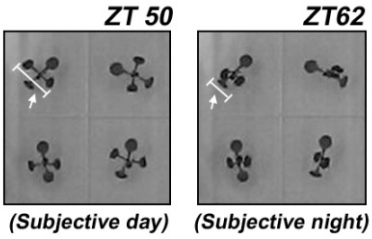
SYNOPSIS

This article is an addendum to the authors' previous article (Kim, J. et al. (2008) Plant Cell 20, 307-319). The instrumentation and software described in this article were used to analyze the circadian leaf movement in the previous article. Here, we provide detailed and practical information on the instrumentation and the software. The source code of the LMA program is freely available from the authors.

The circadian clock regulates a wide range of cyclic physiological responses with a 24 hour period in most organisms. Rhythmic leaf movement in plants is a typical robust manifestation of rhythms controlled by the circadian clock and has been used to monitor endogenous circadian clock activity. Here, we introduce a relatively easy, inexpensive, and simple approach for measuring leaf movement circadian rhythms using a USB-based web camera, public domain software and a Leaf Movement Assay (LMA) program. The LMA program is a semi-automated tool that enables the user to measure leaf lengths of individual Arabidopsis seedlings from a set of time-series images and generates a wave-form output for leaf rhythm. This is a useful and convenient tool for monitoring the status of a plant's circadian clock without an expensive commercial instrumentation and software.



Keywords: Arabidopsis thaliana, circadian clock, leaf movement, software

Introduction

The circadian clock regulates endogenous rhythms with a period of approximately 24 hours, and helps organisms adapt to circadian changes in environmental light and temperature. Plants have evolved to utilize their circadian clocks to stimulate cyclic activity of key molecules in various physiological processes such as photosynthesis, stomata opening, photoprotection, hypocotyl and petiole elongation, and leaf movement (reviewed in Yakir et al., 2007).

Circadian clock activity in plants can be monitored by several rhythmic outputs including clock-controlled physiological responses as well as clock-controlled gene (CCG) expression. In the early stages of the Arabidopsis circadian clock experiments, RNA gel analysis for cyclic CCG expression was used to determine the status of the plant circadian clock despite the fact that this method requires the collection of multiple time-series samples and is labor intensive (Millar and Kay, 1991; Zhong and McClung, 1996; Heintzen et al., 1997). Our present knowledge about the plant circadian clock is largely due to the introduction of CCG promoter-driven luciferase (LUC) into Arabidopsis, which provides a powerful tool for monitoring circadian clock activity in plants (Millar et al., 1992). This tool is non-invasive, visible in real time, and highly reproducible, which allowed isolation of a number of clock components in Arabidopsis using high-throughput genetic screening (Millar et al., 1995). Yet, the bioluminescence measurement system requires a genetic cross to deliver the CCG promoter-driven luciferase (LUC) into the plant of interest and sometime be very time-consuming. For example, the recent development of a genome-wide insertional knock-out pool in Arabidopsis (Alonso et al., 2003) provides valuable resources to identify the circadian functions of specific genes of interest, overcoming the limitation of classic forward genetic screening. Analysis of circadian rhythm for these individual insertional lines requires a more direct assay system than the bioluminescence assay. Leaf movement rhythm is an alternative option for examining the effects of an individual gene on the circadian clock.

Leaf movement rhythm has been used to investigate clock activity in plants ever since it was shown to be the first evidence for the existence of circadian rhythms even under constant environmental conditions (de Mairan, 1729). Plant leaf movement is a result of differential rates of cell expansion in abaxial and adaxial cells in petioles which adjust their turgor pressure resulting in leaves rising and falling during subjective night and day, respectively (Figure 1). Although the physiological importance of this rhythmic movement has still not been determined, this activity shows robust rhythmic activity with approximate 24 h periods for more than 8 days even under constant environmental conditions, which indicates that leaf movement is a typical circadian clock-controlled rhythmic output (de Mairan, 1729; Kim et al., 2008, Figure 4).

Measurement of leaf movement as a rhythmic output of the circadian clock requires three basic steps: 1) taking and collecting plant images at regular intervals, 2) extracting the numeric angles of the leaf petiole or leaf length on the horizontal or vertical axis of the collected images, and 3) estimating the period, phase, and amplitude from the time-series numeric data.

Several systems have been developed to support analyses of the three basic steps and have been used to investigate Arabidopsis clock function in several previous studies (Millar et al., 1995; Onai et al., 2004; Edwards et al., 2005). However, these integrated systems all require expensive hardware modules or commercial software. In addition, these automated systems require delicate control of environments in order to acquire a series of images with good quality and a large number of plants to generate statistically convincing data.

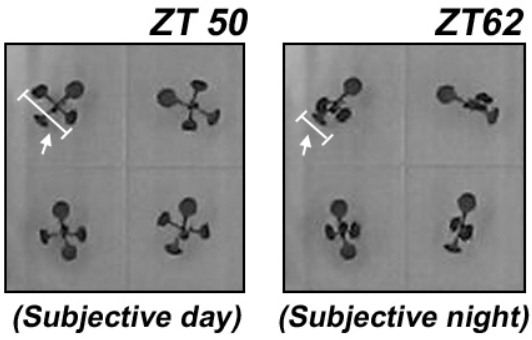


Figure 1. Image capture of an Arabidopsis seedling. Arabidopsis seedlings were entrained under 12h light-dark cycles for 10 days and then transferred to a controlled growth chamber with 24h continual white light and constant temperature. The images were captured at ZT 50 (subjective day) and ZT 62 (subjective night) after transfer into continual light. Bars (arrowed) indicate the length of leaves at the given time point.

Here, we have developed a relatively easy and simple method to measure the rhythm of circadian leaf movement in Arabidopsis plants using a USB-based web camera, non-commercial image capture software, and a Leaf Movement Assay (LMA) program. This system supports the measurement of leaf movement rhythms for approximately 36 plants in each trial, enabling the user to analyze plants with four different genotypes at the same time. In addition, the LMA program can be adapted to enable semi-automated analysis that allows users to change parameters in the middle of analysis, so that even images of low or variable quality can be used. Thus, this system helps users to measure circadian rhythm in plants easily without expensive machines or commercial software.

Experimental set-up and the software

A. Plant growth condition

Arabidopsis seeds were imbibed at 4℃ for 3 days to promote even germination. Thirty six seeds were sown on each square plate (12.5X12.5cm) containing growth media (1/5 Gamberg's B5 media supplemented with 1% sucrose). The seedlings were entrained in a controlled growth chamber (12h light-dark cycles; 23℃ constant temperature; 100-150 μmol m^{-2} s^{-1} of photon flux from F48T12/CW/VHO light bulbs, Philips, Eindhoven, Netherlands) for 10 days, and then transferred into constant light conditions with a lower photon flux (20-25 μmol m^{-2} s^{-1}; FCL32SD/30, Byul-pyo, Seoul, Korea).

B. Image acquisition

Images of seedlings were recorded hourly for 6-8 days using a USB-based camera (a 640 x 480 resolution KDC205, Kocom, Seoul, Korea) and the Dorgem software (http://dorgem.sourceforge.net/). The camera was positioned approximately 30 cm above the plate containing the plants in the growth chamber and connected to a computer (Desktop PC with the Microsoft windows XP system) outside the chamber. Dorgem was used to capture and save images in JPEG format at regular time intervals. Time-series capturing in the Dorgem software required to set the file name as a standard name and special characters (e.g leaf%d%g). The brightness and contrast of images were adjusted to adequately distinguish plants from their background.

C. Measurement of plant leaf lengths from a time-series of images

In this method, the collected images must be converted into image files in order to be recognizable by the LMA program. We created files with the bitmap (BMP) file format with 640X480 resolution, 8-bit grayscale and used a series of file names with a standard name and sequential numbering (e.g. leaf 0 to leaf 100).

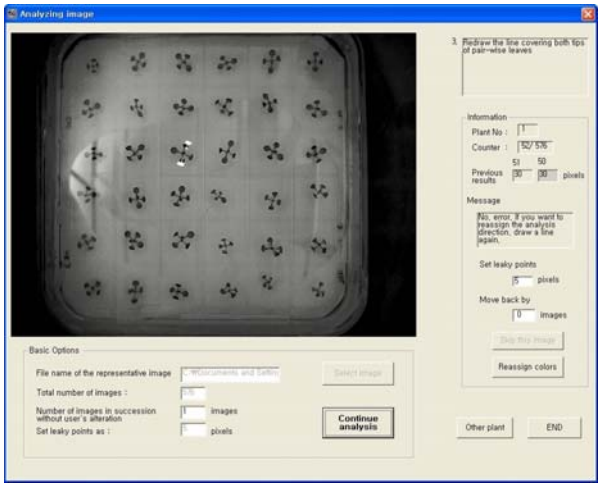


Figure 2. A snapshot of the analysis process for measuring tip-to-tip distances in single seedlings. The LMA program estimates the tip-to-tip distance for one plant in the 52nd image by searching the leaf region and drawing a white line at the 30 degree arc. At the margin of the first and second leaves, the cursor tried to search for leaf color throughout the range of the 30 degree arc by increasing the radius up to the leaky point value. There is no error message in the message window, so clicking the "Continue analysis" button or pressing the space key will proceed to analysis of the next image.

This file conversion can be carried out by many image viewing programs such as Alsee (http://www.altools.com/Default.aspx) and FastStone Image Viewer (http://www.faststone.org/download.htm).

Using the collected images, the LMA program enables users to measure the tip-to-tip distance of pair-wise leaves in a semi-automated manner. The LMA program was written using Visual Basic 6 (Microsoft. Corp., Santa Rosa, CA) and is functional with the Windows XP OS.

Use of the LMA program involves four steps: 1) the establishment of basic parameters for analysis of images from a representative picture, 2) analysis of tip-to-tip distance between a pair of leaves in a single image, 3) management of error messages during the analysis process, and 4) moving on to the next plant or exiting the program. The detailed procedure for measuring leaf distances is as follows:

1. Establishment of basic parameters for analysis of images from a representative picture.
- Open a representative file to set leaf and background colors.
- Click two pixels sequentially to acquire color information for the leaf and for its background region for the plant to be measured.
- Draw a line between the emergences of the 1st and 2nd leaves. This line should cover the tips of both leaves to be measured.
- Open the first image to be measured (the representative file is chosen as a default).
- Enter the total number of images (enter a number at least as high as the total number of images in the time series; 576 is the default).
- Enter the number of images to be processed automatically in succession without alteration by the user (1 is the default).
- Set the leaky point (5 is the default). This value is the number of pixels through which the cursor will search for leaf regions farther, even though the cursor fails to find leaf regions. This value is dependent on image quality; in general, high values are used for bad images and low values for good images.

2. Analysis of tip-to-tip distance between a pair of leaves in a single image
- The LMA program determines leaf length by the distance between the tips of two leaves on a single plant. This can be accomplished by a function in which the cursor will check the color of the pixels in a 30 arc region from the center of the plant towards the direction of leaf emergence. If the cursor finds the leaf color, the arc radius increases by one. If the cursor fails to find any leaf color in the arc, the cursor will try to find the leaf region by increasing its radius up to the leaky point number. This function enables reliable assessment of leaf length even when the images being analyzed are unclear or blurry.
- Figure 2 shows how this function is used on an image upon analysis.
- If no errors occur for this function, the program continues on to analyze the next image.

3. Management of error messages during the analysis process.
- A user will encounter error messages from the program when the leaf length is unreasonably high or low compared to previous readings. This can result from a failure to distinguish the leaf region from its background or by a change in the leaf's emergent direction.
- In such cases, a user can reassign the leaf and background color, and the direction of leaf emergence.
- Otherwise, a user can go back a couple of images and start to measure the leaf length again.
- If a user chooses not to address the error message, he/she can skip over the current image file by pressing the "skip this image" button. In that case, the program estimates the current leaf length using the average of the images before and after that one.

4. Moving on to the next plant or exiting the program.
- Upon finishing measuring the leaf length of one plant across the time course, a user can move on to the next plant or exit the program.
- By clicking the button "other plant," a user can reset the color information for the leaf and background and assign the leaf's emergent direction for the plant to be measured again.
- When a user exits the LMA program, the results will be saved in a file called "result.txt" in the same folder as the LMA program.
- The results file includes the values for the leaf lengths for each plant over the time course (Figure 3).
- If the program was not exited normally, the user can get the results from the "temp.txt" file.

D. Analysis of leaf movement rhythm.

The results file generated by the LMA program can be plotted directly (Figure 4) or easily converted to a file format that can be imported into a circadian rhythm analysis program such as the

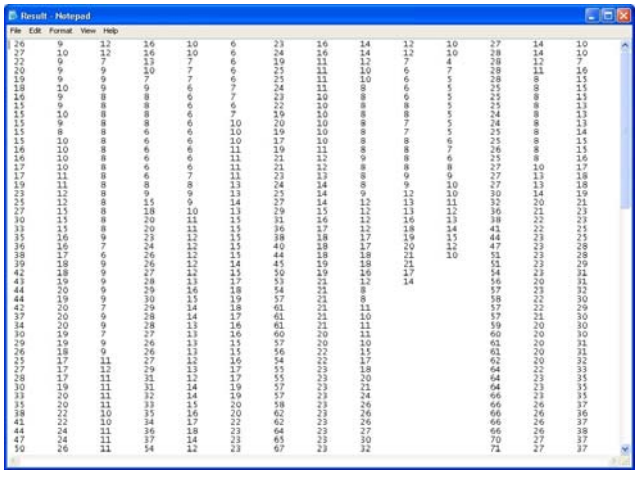


Figure 3. A representative results file for the leaf movement assay using the LMA program. The output results were stored as a file called "result.txt", which is opened using the Notepad program. Numbers indicate the number of pixels between the tips of the first and second leaves. The rows display the pixel numbers for each plant in a single image. The top row displays the first image and the last row displays the last image taken. Each column represents one plant and shows all the readings taken for that plant.

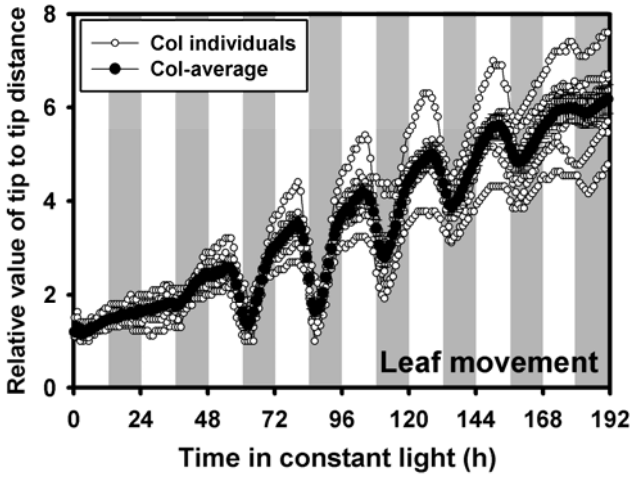


Figure 4. The measurements of Arabidopsis leaf length on the horizontal axis plotted against time. Arabidopsis seedlings were entrained in 12h light-dark cycles for 10 days and then transferred to continual light. The leaf images were taken with a USB camera above the growth plate every hour for 8 days. The leaf length was calculated by the Leaf Movement Assay (LMA) system and normalized to the minimum value of leaf length over the time course. Measurements are shown as the individual (open circles) and average (closed circle) values of the seedlings' horizontal leaf lengths.

Biological Rhythm Analysis Software System (BRASS) program (available from www.amillar.org). The parameters of the circadian clock such as period, amplitude, and phase can be evaluated using the FFT-NLLS function (Plautz et al., 1997). Detailed procedures for using the BRASS program have been published elsewhere (Edwards and Millar, 2007) and can also be found online (www.amillar.org).

Prospects

This system was designed to measure the rhythm of leaf movement without needing to purchase any expensive hardware or software. Users can implement this system easily without any special expertise. Despite its simple design, this system requires three or four independent programs to evaluate leaf movement circadian rhythms. A valuable next step would be to write software to integrate all these functions in order to facilitate analysis. In addition, it would be helpful to develop a system to manage multiple USB cameras to capture images in real time, which would allow a relatively high throughput analysis in order to screen candidate circadian clock gene mutants.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental File 1. The source file for the current version (2.51) of Leaf Movement Assay program (Source file - LMA 2.51.exe – the compressed file)

Supplemental File 2. The install file for the Leaf Movement Assay program (Setup - LMA 2.51 – the compressed file)

Supplemental File 3. The executable file of Leaf Movement Assay program (LMA 2.51).

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