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TITLE:

Using Changes in Leaf Transmission to Investigate Chloroplast Movement in *Arabidopsis thaliana*

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SUMMARY:

Many plant species change the positioning of chloroplasts to optimize light absorption. This protocol describes how to use a straightforward, home-built instrument to investigate chloroplast movement in *Arabidopsis thaliana* leaves using changes in the transmission of light through a leaf as a proxy.

ABSTRACT:

Chloroplast movement in leaves has been shown to help minimize photoinhibition and increase growth under certain conditions. Much can be learned about chloroplast movement by studying the chloroplast positioning in leaves using e.g., confocal fluorescence microscopy, but access to this type of microscopy is limited. This protocol describes a method that uses the changes in leaf transmission as a proxy for chloroplast movement. If chloroplasts are spread out in order to maximize light interception, the transmission will be low. If chloroplasts move towards the anticlinal cell walls to avoid light, the transmission will be higher. This protocol describes how to use a straightforward, home-built instrument to expose leaves to different blue light intensities and quantify the dynamic changes in leaf transmission. This approach allows researchers to quantitatively describe chloroplast movement in different species and mutants, study the effects of chemicals and environmental factors on it, or screen for novel mutants e.g., to identify missing components in the process that leads from light perception to the movement of chloroplasts.

INTRODUCTION:

Light is essential for photosynthesis, plant growth, and development. It is one of the most dynamic abiotic factors as light intensities not only change over the course of a season or day, but also rapidly and in unpredictable ways depending on the cloud cover. At the leaf level, light intensities are also influenced by the density and nature of the surrounding vegetation and the plant's own canopy. One important mechanism that allows plants to optimize light interception under variable light conditions is the ability of chloroplasts to move in response to blue light stimuli[1,2]. Under low light conditions, chloroplasts spread out perpendicular to the light (along

the periclinal cell walls) in a so-called accumulation response, maximizing light interception and hence photosynthesis. Under high light conditions, chloroplasts move towards the anticlinal cell wall in a so-called avoidance response, minimizing light interception and the danger of photoinhibition. In many species, chloroplasts also assume a specific dark position, which is distinct from the accumulation and avoidance positions and often intermediary between those two[3,4]. Various studies have demonstrated that chloroplast movement is not only important for the short-term stress tolerance of leaves[5,6,7], but also for the growth and reproductive success of plants, especially under variable light conditions[8,9].

Chloroplast movement is readily observed in real time in certain live specimens (e.g., algae or thin-leafed plants like *Elodea*) using light microscopy[1]. Studying chloroplast movement in most leaves, however, requires a pretreatment to induce chloroplast movement, chemical fixation, and preparation of cross sections before viewing the samples under a light microscope[10]. With the introduction of confocal laser microscopy, it also became possible to image the 3D arrangement of chloroplasts in intact or fixed leaves[4, 11,12]. These imaging techniques greatly aid the understanding of chloroplast movement by providing important qualitative information. Quantifying chloroplast positioning (e.g., as a percentage of chloroplasts in the periclinal or anticlinal positions in these images or the percentage of area covered by chloroplasts per total cell surface) is also possible but quite time-consuming, especially if conducted at the intervals necessary to capture rapid changes in positioning[10,8]. The simplest way to show whether dark-adapted leaves of a certain species or mutants are capable of chloroplast movement into the avoidance response is by covering most of the area of a leaf to keep the chloroplasts in the dark while exposing a strip of the leaf to high light. After a minimum of 20 min of high light exposure, the chloroplasts in the exposed area will have moved into the avoidance position, and the exposed strip will be visibly lighter in color than the rest of the leaf. This is true for wild type *A. thaliana* but not for some of the chloroplast movement mutants described in more detail later on[13]. This method and modifications of it (e.g., reversing what parts of the leaf are exposed, changing light intensities) are useful for screening large numbers of mutants and to identify null mutants that lack either the ability to exhibit an avoidance or accumulation response or both. However, it does not provide information about the dynamic changes in chloroplast movement.

In contrast, the method described here allows for the quantification of chloroplast movement in intact leaves using changes in light transmission through a leaf as a proxy for overall chloroplast movement: under conditions when chloroplasts are spread out in the mesophyll cells in the accumulation response, less light is transmitted through the leaf than when many chloroplasts are in an avoidance response, positioning themselves along the anticlinal cell walls. Hence, changes in transmission can be used as a proxy for the overall chloroplast movement in leaves[14]. The details of the instrument are described elsewhere (see **Supplementary File**), but basically, the instrument uses blue light to trigger chloroplast movement and measures how much red light is transmitted through that leaf at set intervals. More recently, a modification of this system has been described, which uses a modified 96-well microplate reader, a blue LED, a computer, and a temperature-controlled incubator[15].

The option to use a combination of methods, including the optical assessment of leaves for

screening, followed by measuring dynamic changes in transmission and the use of microscopy, has greatly aided our understanding of both the underlying mechanisms and the physiological/ecological significance of chloroplast movement. For example, it led to the discovery and characterization of various mutants, which are impaired in specific aspects of their movements. For example, *A. thaliana phot 1* mutants lack the ability to accumulate their chloroplasts in low light, while *phot 2* mutants lack the ability to perform an avoidance reaction. These phenotypes are due to an impairment in two respective blue light receptors[16,17,18]. In contrast, *chup1* mutants lack the ability to form proper actin filaments around the chloroplasts which are essential to move the chloroplasts into the desired position within a cell[11,19]. In addition to mutant studies, researchers have assessed the effects of various inhibitors on chloroplast movement to elucidate the mechanistic aspects of the process. For example, chemicals such as H_2O_2 and various antioxidants were used to investigate the effects of this signaling molecule on chloroplast movement[20]. Various inhibitors were used to elucidate the role of calcium in chloroplast movement[21]. In addition to helping to uncover the mechanisms of chloroplast movement, these methods can be used to compare chloroplast movement in various species or mutants grown in different conditions in an attempt to understand the ecological and evolutionary context of this behavior. For example, it has been shown that the extent of the effects of various mutations in the chloroplast movement pathway are dependent on the growth conditions[7,9], and that sun-adapted plants do not seem to move their chloroplasts much. In contrast, movement is very important for shade plants[10,22,23].

This methods paper, focused on the model plant *A. thaliana*, describes how to use a transmission device which is an updated version of a previously developed instrument[9]. While this instrument is not commercially available, people with a basic understanding of electronics or the help of engineering or physics colleagues and students will be able to build the instrument using affordable parts and following the detailed instructions (see **Supplementary File**). The open-source platform used to build the instrument has extensive web support and a community forum which offers help should problems arise[24].

The protocol focuses on how to use the instrument to determine changes in leaf transmission in a standard exploratory run that exposes a leaf to a wide range of light conditions and captures the dark, accumulation, and avoidance reactions of *A. thaliana*. These runs can be modified depending on the goal of the experiment and can be used with most plant species. The paper provides examples of transmission data of *A. thaliana* wildtype and several mutants and shows how to further analyze the data.

PROTOCOL:

1. Preparing leaves for a run

1.1. Place 8 *A. thaliana* plants in the dark overnight (> 6 h works for most species) to ensure that the chloroplasts move into their dark position. All replicas start with comparable transmission values.

1.2. Alternatively, place 8 complete leaves into a Petri dish with a moist filter paper at the bottom, close the Petri dish, and wrap it with aluminum foil.

2. Testing if the transmission device works

2.1. Connect the transmission device to a stable power source and press the power switch (ON/OFF button) of the device to reset the instrument (**Figure 1A,B**).

2.2. Connect the iPad to a stable power source, press the Home Screen button to activate the screen, and enter the passcode to log in.

2.3. Press the **Settings** icon, press the **Display & Brightness** icon, press **Auto-Lock,** and select **Never** by pressing this option to ensure that the screen stays on permanently. Otherwise the program will stop running when the screen goes to sleep. Press the **Home Screen** button to return to the main screen.

2.4. Double press the Home Screen button to see which applications are open, and close all of them by swiping them towards the top of the screen. Press the **Home Screen** button to return to the main screen.

2.4.1. Find the ***LeafSensor*** app on the main screen or by swiping left or right. Press the icon of the app to open it (see **Supplementary File**). A green screen with text and white fields will appear to enter the information.

2.4.2. Ensure the word Connected is seen in the lower part of the screen as it indicates that the app is communicating with the transmission device. If the message '**Adafruit NOT Found**' appears, check that the device is plugged in and press the start button on the device again.

2.5. Fill in the first 4 fields on the app page to name the experiment and to set up the conditions for a brief test run without leaves and with the leaf clips open:

2.5.1. Name the experiment (use 8 or fewer uppercase letters or numbers) e.g., by typing **TEST** into the field named **Expt Name**.

2.5.2. Choose how many different blue light intensities will be used in the experiment, e.g., by typing **3** into the field named **# Light Intensities**.

2.5.3. Choose the blue light intensities for this run (choose an integer between 0 and 3000 and separate each number from the next with a comma; see **Supplementary File** on how to convert these numbers into actual blue light intensities of LEDs) e.g., by typing **0, 100, 1000** into the field named **Blue Intensities**.

2.5.4. Choose the length of time each blue light intensity will shine onto the leaf (separate each number from the next with a comma) e.g., by typing **2,2,2** into the field named **Blue Duration**

(minutes).

2.6. Press **Start Experiment** in the middle section of the screen. In the lower part of the screen 8 hyphens and the message **Starting Experiment** will appear.

2.6.1. Ensure that no light is being emitted from the LEDs for the first two minutes, then weak blue light is being emitted, and after 2 min the blue light intensity is increasing.

2.6.2. Ensure that intense red light is being emitted from the LEDs once a minute for the measurements.

NOTE: As the experiment is running, numbers will appear on the app page for each of the eight sensors, and data will be updated once a minute. Ensure the output numbers from the photodiodes are around 1000-1023 (if the room is dark). An update on the lower left shows how many measurements were done so far e.g., **Done 1 of 6 measurements** (one measurement each minute).

2.6.3. When the experiment is done, check for the appearance of the **Experiment finished** on the lower left of the app page. Now the instrument is ready for a run with leaves.

2.7. Press twice the Home Screen button, swipe out of the app and open it again. Press the ON/OFF button on the instrument to reset it.

3. Setting up leaves in the leaf clips

NOTE: This step has to be done in the dark with a green light source (e.g., place a green filter in front of a light bulb) to avoid inducing chloroplast movement. Alternatively, use very low white light and an extended dark period in the leaf clips. Remember, one part of the leaf clip holds the LED (larger opening), while the other holds the phototransistor (**Figure 1C**).

3.1. If the plants are dark-adapted entire, pick 8 leaves wide enough to cover the LEDs. Otherwise, remove the leaves from the Petri dish. Prepare 8 strips of filter paper about the length of a leaf clip and with a hole punched out at the top to not cover the LED.

3.1.2. Moisten the filter paper and place it onto the leaf clip part that holds the LED. Repeat this for each of the eight leaf clips.

3.2. Place each leaf on the top of the wet filter paper of its leaf clip. Ensure the correct leaf side is facing towards the LED (usually experiments are done with the adaxial leaf surface facing the LED).

3.2.1. Avoid placing the midribs of the leaf on top of the LEDs, and for more consistent results, place similar parts of each leaf (e.g., the widest section of the leaf) over the LED.

3.2.2. Place the other leaf clip part with the phototransistor on top. Use a rubber band to hold the two leaf clip parts together if needed (**Figure 1C,D**).

3.3. Place each leaf clip into its 'boat' and fill the reservoirs with water using a pipette. Ensure the leaf or at least the filter paper touches the water to avoid dehydration of the leaves during the run (**Figure 1D**).

4. Conducting a run

NOTE: For a standard exploratory run, start out with 4 h of darkness (0 µmol photon m^{-2} s^{-1}), followed by 7 h of low blue light (2 µmol photon m^{-2} s^{-1}), followed by 60 min each of 5, 10, 30, 40, 50, 60, 90, 100 µmol photon m^{-2} s^{-1} of blue light. This will induce the leaves to exhibit their dark transmission, induce chloroplast movement into the maximum accumulation, and show different degrees of avoidance response.

4.1. Set up the *LeafSensor* app on the iPad using the steps described below.

4.1.1. Type **EXPLORA1** into the field named **Expt Name**.

4.1.2. Type **10** into the field named **# Light Intensities**.

4.1.3. Type **0,1,60,160,550,750,950,1150,1350,1950** into the field named **Blue intensities**.

4.1.4. Type **240,420,60,60,60,60,60,60,60,60** into the field named **Blue Duration**.

4.2. Press **Start Experiment**. After the first min, the output values (usually between 990 and 820) will appear on the screen. If the values are far off, check if the leaves were placed correctly into the leaf clips.

4.3. When the run is done, ensure that the message **Experiment Finished** appears at the bottom left of the screen. Data will be saved automatically.

4.3.1. Put the screen in the upright position (not horizontal). Two new options will appear on the screen, namely **Save** and **Utilities**.

4.3.2. Press **Utilities** and a list of saved files will appear. Select the file of interest, in this case, **EXPLORA1.**

4.3.3. Below the list of files**,** look for **Selected Expt**: **EXPLORA1**. Press **Email**, enter an **email address,** and the data file will be automatically attached to the message. Press **Send**.

NOTE: If there is a long delay until the files arrive, restart the app and send the file again.

4.4. If a run was aborted, but data up to that point are of interest, press **Save** before selecting

Utilities. After several runs, clean up the memory space: Press **Utilities**, select one file at a time, swipe left next to file, and press **Delete** to remove file.

4.5. Press **Back** to run another experiment or if done, press the Home Screen button, swipe to the main screen, press **Settings**, press **Display & Brightness**, press **Auto-Lock,** and press **2 Minutes.**

5. Data analysis

5.1. Download the file **EXPLORA1** from the email, add extension .csv to the file, double-click on the file. Data will be sorted into a spreadsheet with the data for the eight different sensors sorted into separate columns. The last column shows the time (in seconds) at which the data were collected. Delete the first row under the headings (Sensor1-8) if it contains nonsensical data.

5.2. Set up a master data sheet that will contain the results for each sensor in a separate sheet and that converts the output values into % transmission values using the equations obtained from the calibration of each leaf clip and sensor (see **Supplementary File**).

5.2.1. Copy each data set into a separate data sheet (e.g., column A contains the time; column C contains the data of Sensor1).

5.2.2. Set up column B so that it converts time from seconds to minutes. Set up column D so that it contains the formula to convert voltage to % transmission using the equation from the calibration.

5.2.3. Set up a similar data sheet for each sensor and remember the formula used to convert the voltage output into % transmission values may be different for each sensor.

5.3. Plot % transmission (T) against time, min (**Figure 2**).

5.4. Save the data sheet under a new name so that the master data sheet can be reused.

5.5. To further analyze data (**Figure 2**), calculate **ΔT (%) accumulation** (e.g., change of T at maximum accumulation compared to T at dark), **ΔT (%) avoidance** (e.g., change of T at maximum avoidance compared to T at dark), or **dT/dt (%/h)** (e.g., change in T during the fastest part of the accumulation or avoidance reaction). For further details see [8].

REPRESENTATIVE RESULTS:

The different parts of the transmission device are shown in **Figure 1**. The microcontroller is the control unit of the device and controls the light conditions that the leaves, secured in black leaf clips, are experiencing, and stores the light transmission data it receives (**Figure 1A,B**). A close-up of the control unit of the instrument shows the ON/OFF button, the SD card for data storage capability, the Bluetooth shield (which sends the data to the *LeafSensor* app), and the cables that connect to the LEDs (Light Emitting Diodes) and phototransistors. The microcontroller is

positioned at the base of the instrument, and only the edges are visible in the picture (**Figure 1B**). 3D-printed, black leaf clips hold the leaves, LEDs, and phototransistors in place. A wet filter paper is positioned on the leaf clips part with the LED such that the LED is unobstructed, and a dark-adapted leaf is positioned with the adaxial leaf surface facing the LED. The schematic shows that the LED and phototransistor are positioned on the opposite sides of the leaf. The LED can emit blue or red light. The blue light is used to induce chloroplast movement and is turned off for a brief period every minute during which the red measuring light shines onto the leaf. The phototransistor, positioned on the opposite side of the leaf, detects how much red light is transmitted through the leaf and sends the data to the microcontroller and SD card (**Figure 1C**). The two parts of the leaf clip are assembled, and placed into a 3D-printed 'boat' that is filled with water and helps keep the leaf moist during the experiment (**Figure 1D**).

Figure 2 shows a typical data set in which the % transmission data are plotted against time (min). This particular transmission run involved 1 h of darkness, followed by 3 h of low blue light (2 µmol photon m^{-2} s^{-1}), and 1 h each of intermediate (30 µmol photon m^{-2} s^{-1}) and high blue light (100 µmol photon m^{-2} s^{-1}) intensities. The data show that the transmission in *A. thaliana* decreases at low light intensities (accumulation response), while an avoidance response is induced when light intensities further increased. This is not an all or nothing response and the degrees of changes relative to the dark values depend on the exact blue light intensities. These percent changes in transmission (ΔT) can be calculated using the formulas shown below the data. In addition, the speed of transmission changes (dT/dt) during the initial changes in transmission when an accumulation or avoidance response is triggered can be calculated using the slope of the curve.

The average % transmission values of wild type (WT) (**Figure 3A**), as well as *phot 1* and *phot 2* mutant *A. thaliana* leaves (**Figure 3B**) during 19 h long runs are shown. Such extended, exploratory transmission runs are helpful to establish which blue light intensities to use in future runs. The leaves were first exposed to 4 h of darkness, and consistent transmission values indicate that the leaves were fully dark-adapted, which will make the data between replicas more consistent. For the next 7 h, the leaves were exposed to low blue light (2 µmol photon m^{-2} s^{-1}). In WT and *phot 2*, the initial fast decrease in transmission is followed by a slow decrease which indicates that the chloroplasts were moving into the accumulation response. Depending on the species used, it may take different amounts of time to obtain the lowest possible transmission. In many cases, a researcher may only be interested in comparing various mutants at a given time point, so the exposure to very low light may be limited to an hour. Compared to WT, *phot 1* shows a reduced accumulation response. The extended exposure to low blue light is followed by a stepwise increase in blue light intensities each hour (5, 10, 30, 40, 50, 60, 90, 100 µmol photon m^{-2} s^{-1}). The % transmission in *A. thaliana* WT and *phot 1* increases with each increase in light intensity, showing that the chloroplasts move into the avoidance response but this is not seen in *phot 2*. The degrees of change in transmission relative to the dark value (ΔT) depend on the exact blue light intensities and may differ depending on the genotype (**Figure 3C**). An example is shown of the speed of transmission changes (dT/dt) during the initial responses in transmission when avoidance is triggered as the blue intensity is increased from 5 to 10 µmol photon m^{-2} s^{-1} (**Figure 3D**). The speed is the same for WT and *phot 1*, while it is very slow for *phot 2* mutants.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the transmission device. Picture of the home-built transmission device with the control unit in the black box on the bottom right and the leaf clips on top and the bottom left (**A**). Close-up of the control unit with the ON/OFF button, the SD card for data storage capability, and the Bluetooth shield for wireless communication. Cables connect the control unit to the Light Emitting Diodes (LEDs) and phototransistors. The microcontroller is positioned at the base of the instrument and only the edges are visible in this picture (**B**). The 3D-printed black leaf clips: on the left the leaf clip part holding the LED is shown, on the right the leaf clip part holding the phototransistor is shown. To set-up a run, a moist filter paper (with a hole the size of the LED) is placed on the clip without obscuring the LED. Then the leaf is placed in the clip covering the LED. The schematic shows that the LED and the phototransistor (PT) are located opposite each other, close to the leaf, when the two parts of each leaf clip are assembled (**C**). Leaf clips are positioned into 3D-printed 'boats' that are filled with water, keeping the leaves and the filter papers hydrated (**D**).

Figure 2: Transmission data of a typical *A. thaliana* leaf. Transmission (T) data for an *A. thaliana* leaf that was exposed to dark for 1 h, followed by 3 h of low light (2 µmol photon m^{-2} s^{-1}), followed by 1 h each of intermediate (30 µmol photon m^{-2} s^{-1}) and high blue light (100 µmol photon m^{-2} s^{-1}). Low light intensities induced the accumulation response, while higher light intensities induced different degrees of an avoidance response. The T levels at dark serve as the baseline (blue line). The % changes in T relative the dark levels (ΔT) e.g., at maximum accumulation or different levels of avoidance (differences to dark T are indicated by the blue arrows) can be calculated. In addition, the speed with which T changes (dT/dt) e.g., during the initial phase of the avoidance response can be calculated from the slope of T plotted against time (indicated by the blue triangle). The equations are shown below the graph.

Figure 3: Chloroplast movement in wildtype and mutant *A. thaliana* leaves. Dark adapted, mature leaves were exposed to dark for 4 h, followed by a 7 h exposure to 2 µmol photon m^{-2} s^{-1}, followed by a step wise increase in blue light intensity each hour (5, 10, 30, 40, 50, 60, 90, 100 µmol photon m^{-2} s^{-1}). Average % Transmission (T) values (n = 20) of WT (**A**) as well as *phot 1* and *phot 2* leaves (**B**). Change in % T relative to the dark values: negative values indicate that the leaves showed an accumulation response, while positive values indicate an avoidance response. The numbers on the right indicate the blue light intensity at which the ΔT data were calculated. The color scheme is the same as in the rest of the figure (**C**). The dT/dt data were calculated as leaves responded to an increase in blue light intensity from 5 to 10 µmol photon m^{-2} s^{-1} and indicate the speed with which % T changed per hour (**D**). Data for **A** and **B** are means, for **C** and **D** means ± SD (n = 20).

DISCUSSION:

The device is extremely easy to use but it is of crucial importance to calibrate each leaf clip set-up of the transmission device independently since the positioning of the LEDs and phototransistors may slightly vary from leaf clip to leaf clip. Ensure the LEDs and phototransistors are inserted stably and re-check the calibration if the data seem off. Avoid getting water onto the

device. The leaves in the leaf clips are placed into 'boats' filled with water to avoid water stress. Place these boats e.g., into a low rimmed plastic container separate from the control unit and do NOT knock them over. Do not unplug or bend the cable connections. Be careful when inserting leaves into the leaf clips and avoid pulling or bending the cables too much.

It is important to dark-adapt the leaves long enough to ensure the initial transmission values are representing the dark position. Check that the values during the dark period in the device have been stable at least 30 min before shining blue light onto the leaves. If they are not, dark-adapt the leaves for a longer period of time before the next run or extend the dark period in the transmission device to monitor how long it takes for the leaves to reach a steady state. Typically, the transmission data are presented as % change in transmission at a given blue light intensity relative to the dark value (ΔT). Hence it is crucial to obtain the correct baseline values.

The exploratory run can be used for any *A. thaliana* mutant (including mutants known to affect other aspects of a plant's physiology e.g., photosynthesis, myosin or uncharacterized mutants) or different species as long as the leaf area is large enough to cover the LED in the leaf clip and the leaves are not too thick. The program can be easily adapted to fill any needs of a researcher e.g., the blue light intensities can be changed within the ranges that have been shown to elicit chloroplast movement (the reaction saturates around 100 µmol photon m^{-2} s^{-1}), the exposure times can be altered, the number of consecutive light conditions can be changed. In addition, leaves can be pretreated before being run in the device e.g., with inhibitors of actin polymerization or signaling pathway components, which is important for researchers who want to fill in the blanks in the signaling pathway regulating chloroplast movement.

Like every method, this one too has its limitations and drawbacks. The procedure relies on changes in the optical properties of leaves, namely how much light is transmitted. Therefore, it works best with relatively thin leaves, while thick leaves often do not allow for sufficient transmission of red light to be detected beyond the noise level. It would be possible to modify the transmission device to increase the red-light intensity shining onto the leaf and increase the sensitivity of the phototransistors by changing the resistors. Since the method only provides an integrated measure of the movements of chloroplast in all cells and cell layers, one may miss some subtle changes e.g., chloroplasts moving in opposite directions may result in no net change of transmission. Especially when working with previously uncharacterized mutants or species, it is important to complement the transmission results with images of chloroplast positioning using microscopy. For example, slow changes in transmission in response to changes in blue light intensity were observed in an *A. thaliana* mutant and could have been due to a range of reasons. Microscopy revealed that cells only had two chloroplasts which were much larger than normal chloroplasts. The mutant was later confirmed to be the chloroplast division mutant *arc6-1*[25].

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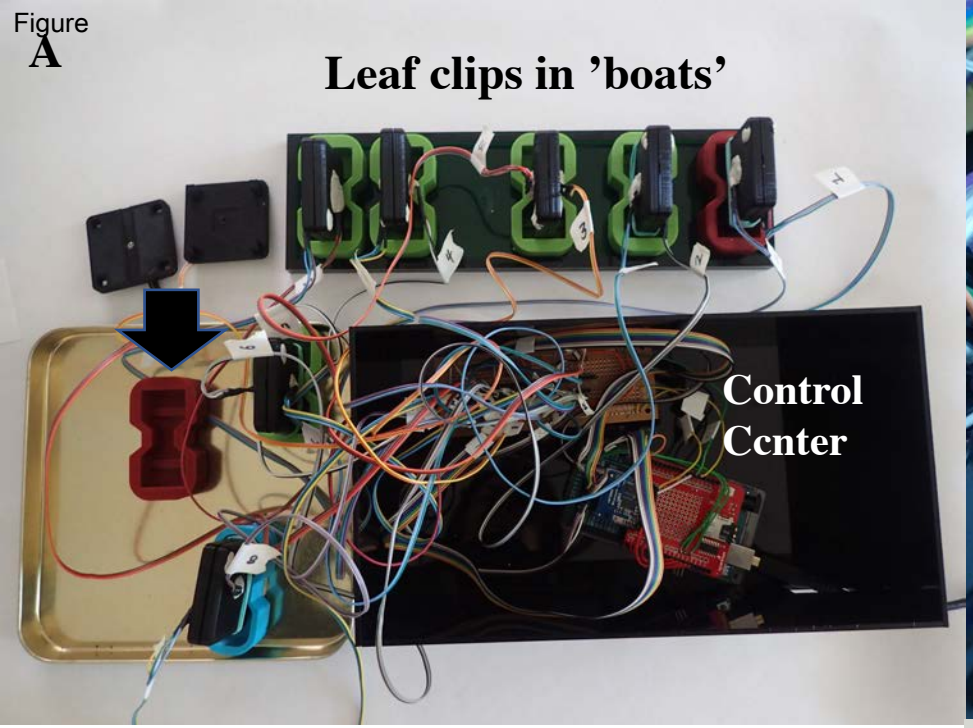
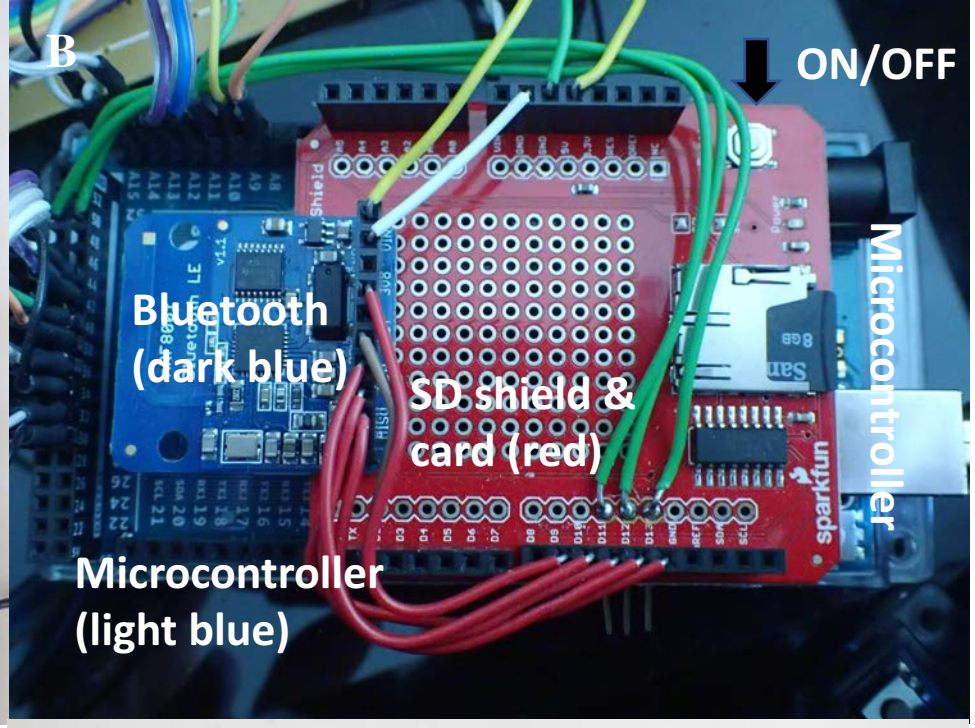
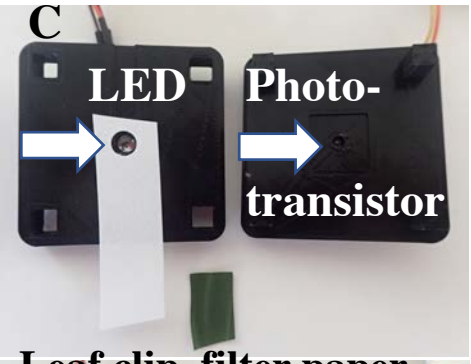
20. Wen, F., Xing, D., Zhang, L. Hydrogen peroxide is involved in high blue light-induced chloroplast avoidance movements in *Arabidopsis*. *Journal of Experimental Botany.* **59** (10), 2891–2901 (2008).

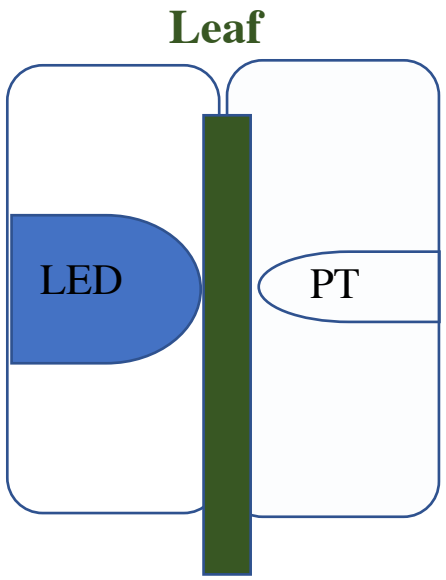
21. Tlalka, M., Fricker, M. The role of calcium in blue-light dependent chloroplast movement in *Lemna trisulca* L. *The Plant Journal.* **20**, 461–473 (1999).
22. Königer, M, Bollinger, N. Chloroplast movement behavior varies widely among species and does not correlate with high light stress tolerance. *Planta.* **236**, 411-426 (2012).
23. Higa, T., Wada, M. Chloroplast avoidance movement is not functional in plants grown under strong sunlight. *Plant, Cell and Environment.* **39**, 871–882 (2016).
24. https://www.arduino.cc
25. Königer, M., Delamaide, J. A., Marlow, E. D., Harris, G. C. *Arabidopsis thaliana* leaves with altered chloroplast numbers and chloroplast movement exhibit impaired adjustments to both low and high light. *Journal of Experimental Botany.* **59**, 2285-2297 (2008).

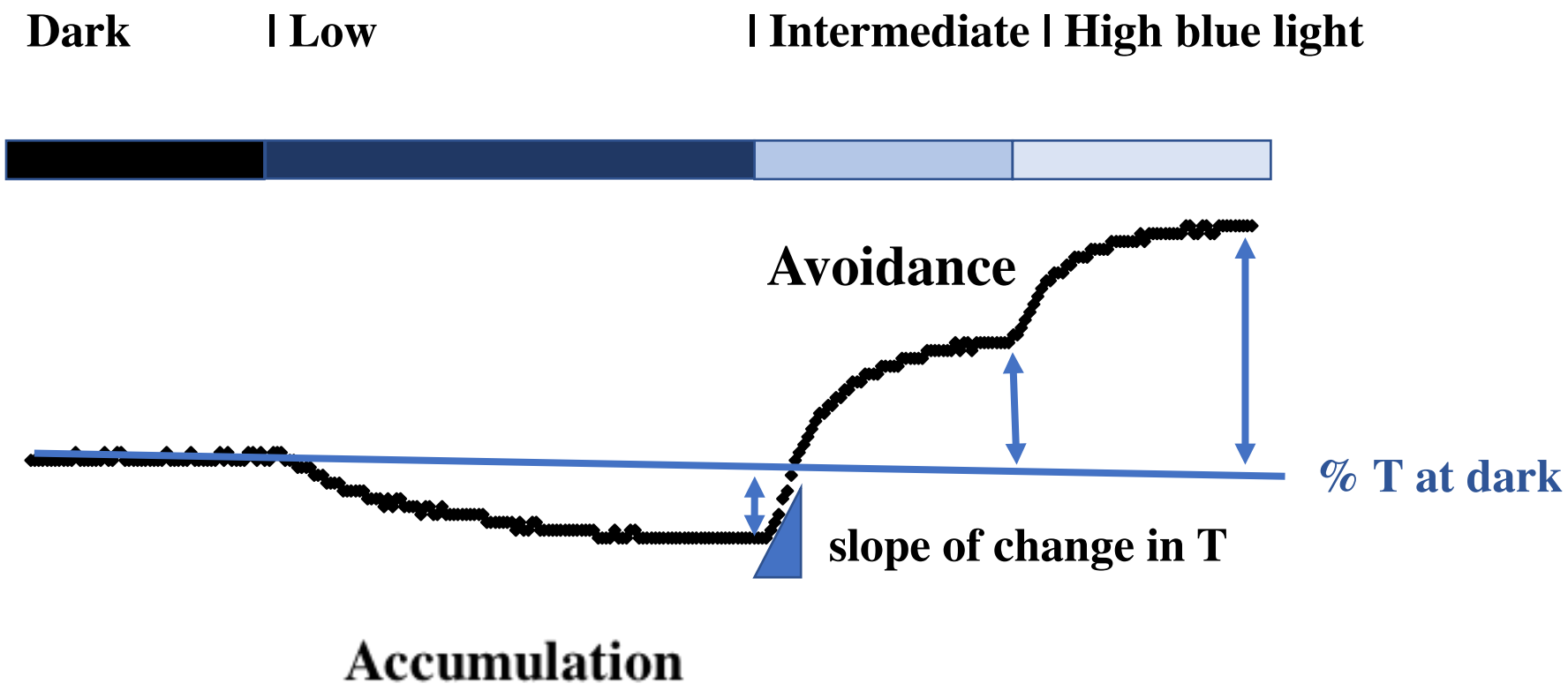
Figure





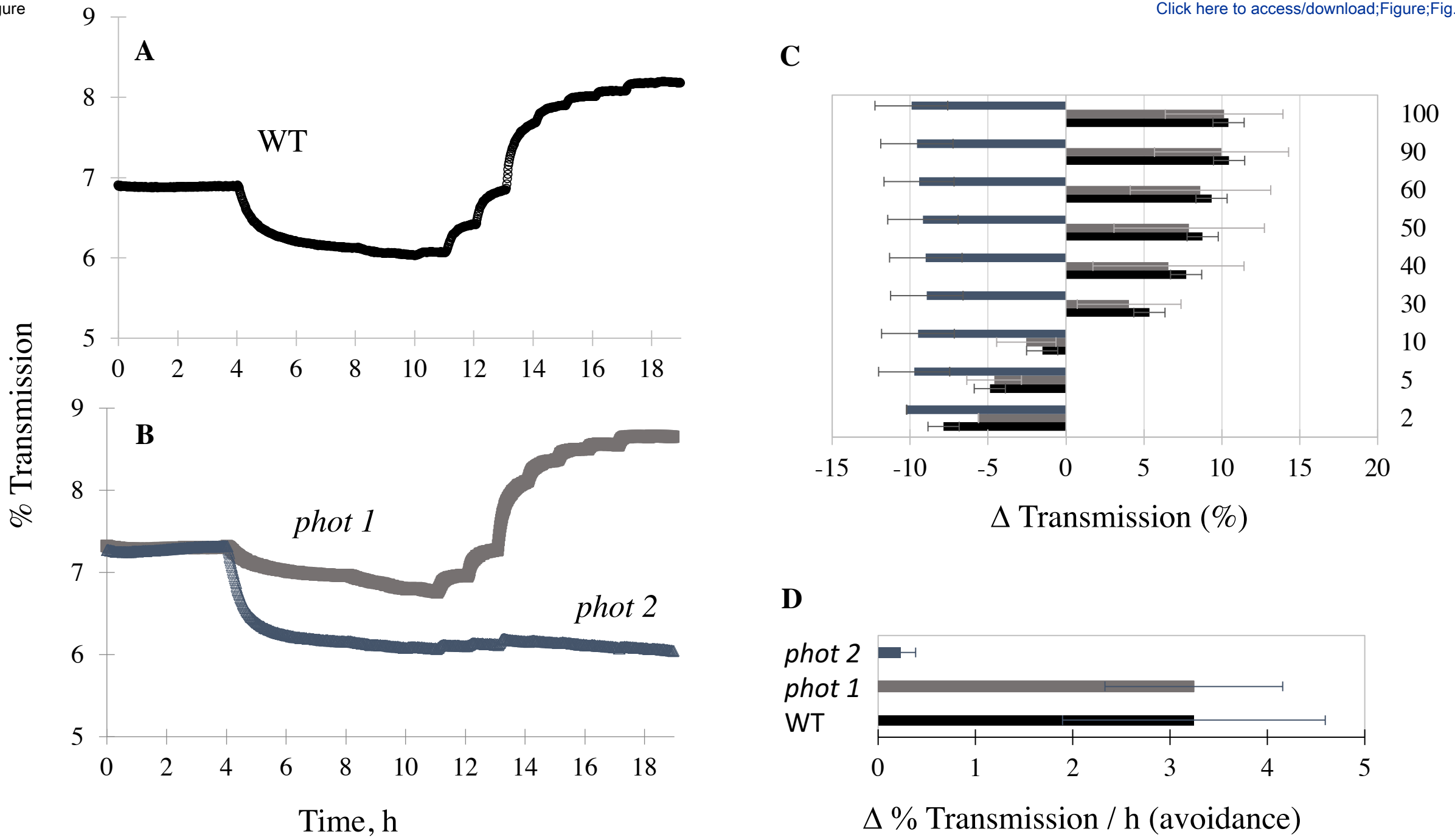


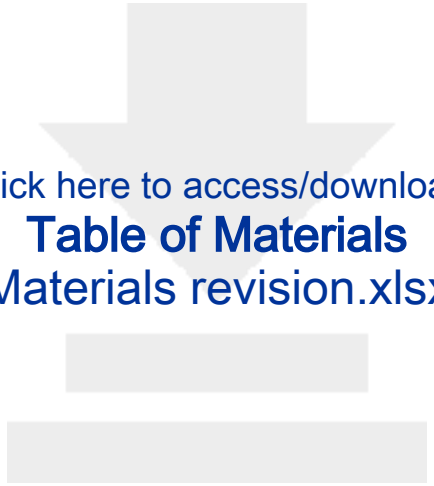




Δ Transmission (avoidance) = (T avoidance - T dark) / T dark
dT/dt (avoidance) = slope of change in T

Figure





Click here to access/download

Table of Materials

Materials revision.xlsx

Dear Dr. Iyer,

We are very pleased to have the opportunity to revise the submitted manuscript.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We did proofread the manuscript.

2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We changed the relevant sentences to avoid personal pronouns (see track changes)

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: SparkFun microSD Shield (is this a specific commercial name of a component in the transmission device?); Excel etc

We replaced SparkFun microSD with SD card, Excel with “data sheet” and Arduino with “microcontroller”.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We added additional information to make the protocol more informative and moved some the non-action items under notes.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We rephrased the instructions and switched everything to imperative tense and included a couple of note: sections.

6. Please sort the Materials Table alphabetically by the name of the material.

We sorted the material in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe the usage of a home-built instrument to measure changes in leaf transmission upon exposure of the leaves with different blue light intensities. These data allow conclusions about the movement of chloroplasts in the cell.

Major Concerns:

The use of the instrument, data collection and data analysis are explained very well and in detail in the manuscript. However, the step by step protocol requires an already assembled instrument. The instructions for building this instrument are given in the supplements. However, these instructions are not detailed enough that an easy building of the instrument is possible. Therefore, from my point of view, the protocol can only be used by other scientists if the assembly of the instrument is also described in more detail.

The other reviewers seemed to think that the instructions were detailed enough. There is a lot of support out there from the Arduino community if people have questions. We added links to plat form and community web site in the manuscript.

Minor Concerns:

Line 175: Here, the authors refer to Figure 1C. I think it should be Figure 1D

We refer now to Figure 1C/D, since both show the leaf clips but in different configurations.

Reviewer #2:

Manuscript Summary:

The video will present an experimental method based on a home-built instrument to investigate chloroplast movement in Arabidopsis thaliana leaves using changes in transmission of light through a leaf as a proxy. This is a very suitable topic for JOVE. The protocol is readable, appears complete and easy to follow. The figures are well selected and nicely illustrate the power of the method. The caveat here is the "transmission device" that is required. This device needs to be home assembled from commercially available electronics parts. While quite simple and well described in the protocol, it's not what biologists do for a living. Typically, the help of the University workshop would be required.

Major Concerns:

As suggested in the manuscript this method could be used to characterize mutants or to carry out a mutant screen. However, changes in

transmission patterns may not in all cases reflect chloroplast movement but, potentially, a lack of chlorophyll or other. For this reason, a cautionary remark should be added that in specific cases (eg new mutants identified using the technique) chloroplast movement should be directly verified by optical microscopy.

We agree and added a section at the end of the discussion, making it hopefully clearer that microscopy is essential to back up the transmission results:
Especially when working with previously uncharacterized mutants or species, it is important to complement the transmission results with images of chloroplast positioning using microscopy. For example, slow changes in transmission in response to changes in blue light intensity were observed in an A. thaliana mutant. Microscopy revealed that cells only had two chloroplasts which were much larger than normal chloroplast. The mutant was confirmed to be the chloroplast division mutant arc6-1[25].

Minor Concerns:
Line 36: "…depending on cloud cover…". Maybe "time of day", "shading vegetation" or others should also be mentioned.

We expanded on this idea:
Light is essential for photosynthesis, plant growth, and development. *It is one of the most dynamic abiotic factors as light intensities not only change over the course of a season or day, but also rapidly and in unpredictable ways depending on cloud cover. At the leaf level, light intensities are also influenced by the density and nature of the surrounding vegetation and the plant's own canopy.*

Lines 38-40: "face" / "profile position" are typically referred to as periclinal / anticlinal position.

We changed the wording following the suggestion.

Line 40: "chloroplast move towards the edges of the cell (profile position)". I'm not sure this should be called the edge of the cell as it's rather a surface. Better formulation might be "alignment along the anticlinal cell wall".

We made the suggested change.

Reviewer #3:
Manuscript summary:
The manuscript entitled "Using changes in leaf transmission to investigate chloroplast movement in Arabidopsis thaliana" by Königer, et al describes a home-built instrument to detect the changes in leaf transmission as a proxy for chloroplast movement. The authors exposed the leaves to blue light intensities to quantify the dynamic changes in leaf transmission. Based on the position of the chloroplasts, the accumulation response and avoidance response were quantified. Further, as a proof of concept the authors carried out a comparative study of using wild-type Arabidopsis and phot 1 and phot 2 mutants exposed to different light intensities. The authors successfully demonstrate differential chloroplast movements in response to light intensities. The study is useful for researchers who are working in plant sciences specifically those working in stress biology and 'light' biology. The manuscript is technically sound.

Additionally, I would suggest the citation of a few other relevant articles in the introduction section of the manuscript.

We added a few extra sections eg about other methods to the introduction and added 15 additional articles.

Reviewer #4:
Manuscript Summary:
Leaf light transmission has been used as a proxy for monitoring light-induced changes in chloroplast movements for many years. In fact many published research papers on the topic of chloroplast movements routinely include plots of leaf light transmittance similar to the example shown in this paper. Light-dependent chloroplast movements were first described in the 1850's but received little attention until researches adopted genetic and molecular approaches to study them.

This paper describes what they call a simple method to measure leaf light transmittance. While the home-made instrument and associated methodology does indeed provide a good way to monitor blue light-dependent chloroplast movements, simple is not a word I would use to describe it. I think straightforward would be a better way to describe the system. For the following reasons:

1) Building the electronic part of the instrument is very likely to be intimidating to those that have no experience assembling electronic components. However, the step-by-step instructions are straightforward and could be followed by someone with little to no experience with electronics.

2) Likewise, linking together the electronics, leaf clamp system, iPad, etc. is straightforward with the detailed instructions but even that is not exactly simple.

3) Similarly, the protocol outlined for using the instrument has many steps that need to be followed exactly to obtain the result in the desired format. It is straightforward if one follows the instructions, but again, not exactly simple.
Overall, because there is a lot to be gained by those who build their own instruments so as a pedagogical exercise, I strongly support the publication of the paper. In spite of not fitting my criteria for simplicity, the system described can be very useful and would allow many

people to experience on of the more amazing and quite rapid plant responses that is largely overlooked by most people, including plant biologists.

We appreciate the nuanced response. We changed the wording from simple to straightforward. We changed this paragraph and added this info:
While this instrument is not commercially available, people with a basic understanding of electronics or the help of engineering or physics colleagues and students will be able to build the instrument using affordable parts and following the detailed instructions (see Supplemental Info). The open-source platform used to build the instrument has extensive web support and a community forum which offers help should problems arise[24].

Minor Concerns:
I think it would be good if the authors were to mention and provide a few refences to some of the other systems researchers have used to monitor and chloroplast movements in leaves. For example, the simplest method that has been used takes advantage of the fact that one can actually see the effect of light-induced chloroplast movements by covering a leaf with a mask that blocks light from part of a leaf and allows light to reach other parts of a leaf (they use stripes). Removing the mask after about 20-30 min of light exposure can result in lighter green areas that had been irradiated compared to the darker green of the areas that were shielded by the mask. The Wada lab to be the first to identify that the phototropin 2 photoreceptor1 regulates the high light avoidance response and then went on to use the method to identify many other chloroplast movement mutants. Basically, they took Indeed, the cover of the issue of Science1 that their first paper was published in has an image of an Arabidopsis leaf with the Japanese symbol for light in the leaf that was created by using covering the leaf with a stencil of the symbol. It is hard to get much simpler than that. The strip assay does not provide kinetic information but it is an effective and is actually simple. The only caveat is that one needs to use thin-leafed species to be able to see sufficient changes in the green hue. Arabidopsis is great for the technique.

It might also be nice to point out that labs have started to use multicell plate readers to make the leaf transmittance measurements. Plate readers have come into wide use in many fields of biology and are often used in various college teaching labs. Most plate readers are relatively easy to use and citing some of the papers describing their use for measuring chloroplast movements may be appreciated by those less inclined to make their own instrument.

1. Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science 291: 2138-2141

We added additional descriptions of other methods and more references (25 now instead of 14):
The simplest way to show whether dark-adapted leaves of a certain species or mutants are capable of chloroplast movement into the avoidance response is by covering most of the area of a leaf to keep the chloroplasts in the dark while exposing a strip of the leaf to high light. After a minimum of 20 min of high light exposure, the chloroplasts in the exposed area will have moved into the avoidance position and the exposed strip will be visibly lighter in color than the rest of the leaf. This is true for wild type A. thaliana but not in some of the chloroplast movement mutants described in more detail later on[13]. This method and modifications of it (e.g. reversing what parts of the leaf are exposed) are useful for screening large numbers of mutants and to identify null mutants, that lack either the ability to exhibit an avoidance or accumulation response or both. However, it does not provide information about the dynamic changes in chloroplast movement.

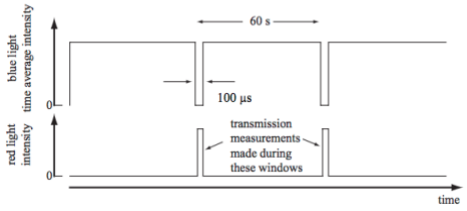
More recently a modification of this system has been described which uses a modified 96-well microplate reader, a blue LED, a computer, and a temperature-controlled incubator[15].

We hope we addressed all the concerns raised on the review.

Best, Martina Königer

I. System overview

We are using an updated version of a photometric instrument that allows for the quantification of transmission changes due to chloroplast movement (Berg et al. 2006; http://academics.wellesley.edu/Physics/Rberg/papers/LogoChipLeaf.pdf). The basic idea of the instrument is to use a blue/red LED to shine blue light of defined intensity onto a leaf and induce chloroplast movement, before switching to red light for a brief moment to measure how much of the red light is transmitted through the leaf. The red light is detected by a red-light sensitive phototransistor that is positioned on the opposite side of the leaf. The original instrument was controlled by a LogoChip.



The updated instrument uses an Arduino microcontroller and readily available Arduino accessories, but the basics are the same. The extensive Arduino documentation, accessibility of components and low cost mean that this instrument can be relatively easily built. The instrument is connected to an iPad, and an app (LeafSensor.ipa) is used to set-up the experimental protocol and to record data. The data are also stored on an SD card as a backup. Leaf, LED and phototransistor are held in place by a 3D-printed leaf clip, which is positioned in a 3D-printed boat to which water can be added to keep the leaf from drying out. The entire set-up allows for 8 leaves to be tested.

II. Instructions for building the instrument

1. List of parts

Arduino Mega 2560 R3 SKU: DEV-11061 (SparkFun) https://www.sparkfun.com/products/11061

SparkFun microSD Shield SKU: DEV-12761 (SparkFun) and **microSD card** for extra memory https://www.sparkfun.com/products/12761

SparkFun microSD Shield communicates using the SPI interface https://www.arduino.cc/en/Reference/SPI and uses four digital I/O pins https://learn.sparkfun.com/tutorials/microsd-shield-and-sd-breakout-hookup-guide

Adafruit Bluefruit LE - Bluetooth Low Energy (BLE 4.0) - **nRF8001** Breakout - v1.0
https://www.adafruit.com/product/1697

USB Cable to power the Arduino
https://www.amazon.com/AmazonBasics-USB-2-0-Cable-Male/dp/B00NH11N5A

2. Wiring

• Bend out or cut off pins D11-13 on the red **SparkFun microSD shield** before plugging in the shield.

• Solder wires into the holes for the D11-13 on the **SparkFun microSD shield board** and connect them as follows to the **Arduino Mega 2560:**

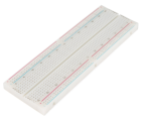
SparkFun 11 -> Mega 51 (MOSI)
SparkFun 12 -> Mega 50 (MISO)
SparkFun 13 -> Mega 52 (CLK)

• Next connect **Bluetooth nRF8001** to **SparkFun microSD shield**:

nRF8001	SparkFun microSD shield (modified)
CLK	-> 13
MISO	-> 12
MOSI	-> 11
REQ	-> 10
RDY	-> 2
RST	-> 9
RESET	-> 8
GND	-> GND
Vin	-> 5V

• Use a breadboard to connect the LEDs, phototransistors, and resistors for the 8 sensors:

External board to connect LEDs and phototransistors
https://www.sparkfun.com/products/12615

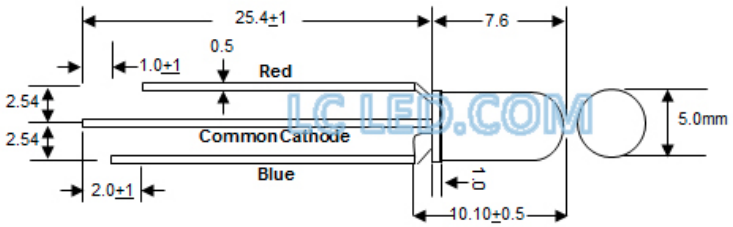


5 mm Blue/Red LED: *LC* LED-N500TBR4D
http://www.lc-led.com/View/itemNumber/150

Electro-optical Characteristics (Ta = 25^{0}C)

PARAMETER	SYMBOL	CONDITIONS	MIN.	TYP.	MAX.	UNIT
Forward Voltage (Blue)	V_F	I_F = 20mA	3.0	3.0	3.8	V
Forward Voltage (Red)	V_F	I_F = 20mA	1.7		2.6	V
Reverse Voltage	V_R	I_R = 100mA			5	V
Dominant Wavelength (Blue)	I_D	I_F = 20mA	464		475	nm
Dominant Wavelength (Red)	I_D	I_F = 20mA	624		635	nm
Viewing Angle	2q1/2	I_F = 20mA		30		Deg.
Luminous Intensity (Blue)	I_V	I_F = 20mA	1,800		3,200	mcd
Luminous Intensity (Red)	I_V	I_F = 20mA	4,000		6,500	mcd

DEVICE DRAWING



Phototransistor Silicon NPN T-1: https://www.digikey.com/products/en/sensors-transducers/optical-sensors-phototransistors/544?k=480-1951-ND&pkeyword=&FV=ffe00220&mnonly=0&ColumnSort=0&page=1&quantity=0&ptm=0&fid=0&pageSize=25

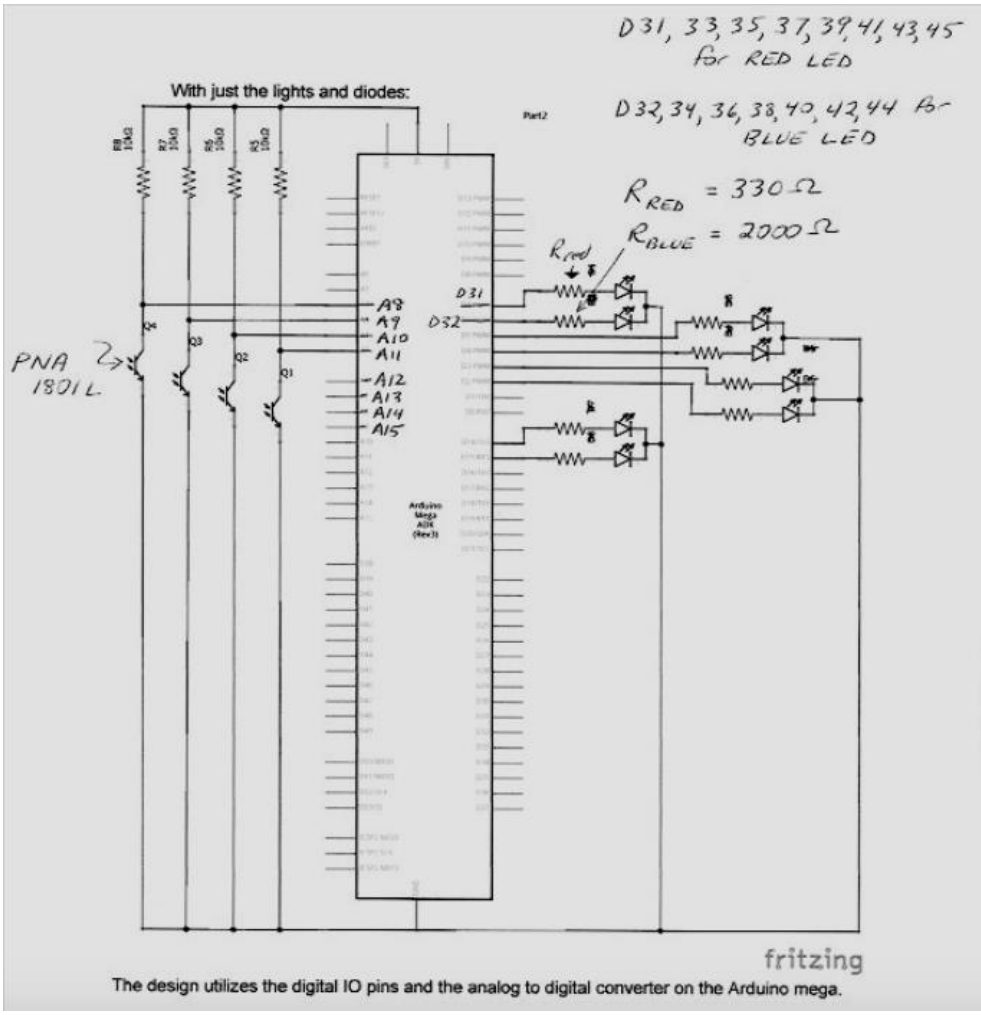
Resistors
resistors (upright) for the red LED lead 330 Ohm 

resistors (horizontal) for the blue LED lead 1K Ohm 

pull-up resistor between the 5V and the phototransistor 10K Ohm 

Important to get a useful range of output values. For more info see:
https://learn.sparkfun.com/tutorials/pull-up-resistors

Wiring scheme:



The design utilizes the digital IO pins and the analog to digital converter on the Arduino mega.

Eight phototransistors (PNA 180IL) are connected to A8-A15 and eight LEDs connected to pins D31-45 on the Arduino Mega 2560 as listed above in the wiring scheme.

Connect 5V to 5V on the red board.
Connect Vin to Vin on blue board.

3. Arduino program

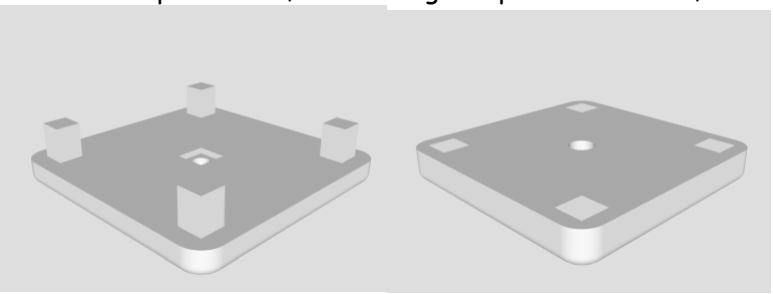
• See Supplemental coding Arduino

• Make sure to install all of the libraries needed for the SD card shield and the BLE peripheral.

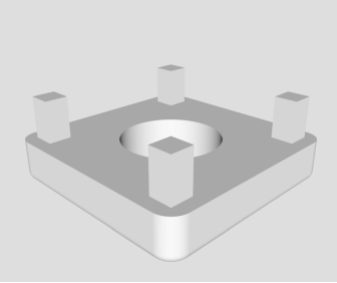
4. Instructions for making leaf clips

Use a 3D printer to make the leaf clips. They need to be made out of black filament (e.g., Black PLA Filament -1.75mm Matter Hackers) otherwise light from the LEDs will be picked up by other leaf clip phototransistors.

There are 2 parts to it, one holding the phototransistor, one the LED.

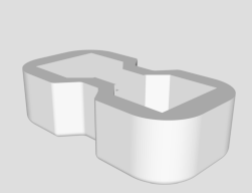


We also printed a clip to hold a light meter sensor that allows us to determine the blue light intensities.



The leaf clips were placed in 'boats' that can be filled with water to make sure the leaves do not dry out in longer runs.

Bend some of the wires so that there is not a cable mess.



5. Instructions for making the box

Black acrylic laser cut, then assembled with acrylic "glue" which dissolves the acrylic, basically chemically welding the sides together.

6. LeafSensor app

Contact Susan Kohler (skohler00@gmail.com) for info about the app.

To install the app:

• Hook up the iPad to your computer and open iTunes. You should see your iPad listed as a device in the panel on the left.

(Delete the old version of Leaf Sensor from the iPad if you have one)

• Drag the **LeafSensor.ipa** file over the iPad in the device list. It should install the app on the iPad.

III. Instructions for determining blue light intensities of LEDs

There may be slight difference in the leaf clips because of the inaccuracies of the 3D printer, the manufacturing of the LEDs, or because of the exact positioning of the LEDs in the leaf clips. Therefore, it is important to label each leaf clip and LED and to calibrate them separately.

• On the iPad, set **Setting** to **Auto-Lock**, **Never;** pick **Expt Name**: eg CAL; choose **Blue Intensities**: 1, 100, 1000, 2000, 3000; choose **Blue Duration (minutes)** 10, 10, 10, 10, 10

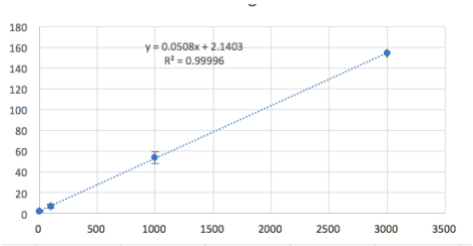
• Attach the part of leaf clip 1 with the LED to the matching leaf clip that holds the light sensor of a light meter (eg LICOR LI-190R Quantum Sensor and LI-250A).

• Press **Start Experiment**, write down the read out of the light meter in μmol photon m^{-2} s^{-1}; switch the leaf clip that holds the light sensor to LED 2; note the read out of the light meter; repeat until all eight LEDs have been measured.

• In an excel sheet plot **Light intensity** (read out of light meter) vs Blue Intensities (1, 100, 1000, 2000, 3000) for each sensor.

• The light intensities have to be similar for the eight set-ups. If they are different, check that you used the correct LEDs and if necessary adjust their position in the leaf clip until they are similar and then secure them in place with glue or patty.

• Average the light intensities for all 8 leaf clips and plot the values against Blue Intensities (1, 100, 1000, 2000, 3000); get a linear regression and the formula that describes the relationship.



In our set-up, the true light intensity in µmol photon m^{-2} s^{-1} = 0.0508 * Blue Intensities + 2.14
1 = 2.2 µmol photon m^{-2} s^{-1}
100 = 7.2 µmol photon m^{-2} s^{-1}
1000 = 53 µmol photon m^{-2} s^{-1}
3000 = 155 µmol photon m^{-2} s^{-1}

IV. Instructions for calibrating phototransistors to convert voltage output into % transmission

Use filters e.g., https://www.barbizon.com/product_info/rosco-roscolux-light-grey.html?attr={8}161 or https://www.barbizon.com/search/browse/?psearch=site&keywords=Kelly+Green to achieve certain transmissions between 1% and 15%. To figure out which filters and filter combinations to use, set up a light source of known intensity e.g., 100 µmol photon m^{-2} s^{-1} (measured at a set distance from the light source with a light meter). Place filters in front of light source and determine the light intensity, which will allow you to determine the % transmission e.g., if the light intensity with filter is 10 µmol photon m^{-2} s^{-1}, then the transmission is 10%. Select at least 4 different filter combinations that generate a range of % transmissions:

3* #3 = 2.8%T
2* #3 = 7.9%T
3* #4 = 11.5%T
2* #4 = 15.9%T
#3 = our grey filter 1
#4 = our grey grey filter 2

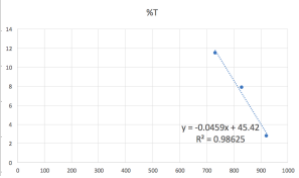
Our instrument loses it linearity if %T is above 16.

If you are using several filters at the same time, tape them together and avoid touching them in the area that will be inserted into the leaf clip. Make sure the filters stick out on top and the bottom so that you can be sure that they were inserted correctly and are placed between the LED and the phototransistor.

Place the filters, one after the other, into each leaf clip, turn on program with BL at e.g., 1000 and write down the Voltage output that you see on the app screen.

Plot the data with Voltage on the X-axis and % transmission on Y-axis. Fit a linear regression line and get the formula and R^2. If the different leaf sensors are similar, average the values and use the formula in the excel sheet that will convert Voltage into %T. If not, keep the calibrations separate for each leaf sensor and use the respective formulas for each Excel sheet.

Sensor 5	%T
829.8	7.9
921.25	2.8
732.25	11.5



V. Excel sheet for data analysis

Make a master Excel sheet that has the results of the calibrations for each sensor in a separate sheet. Copy each data set into the respective data sheets: column A, contains the time; column C contains the data. Set up column B so that it converts time from seconds to minutes. Set up column D so that it contains the formula to convert voltage to % transmission using the equation from the calibration. The formula may be different for each sensor:

A	B	C	D	E
Time, s	Time, min	Sensor 1	% Transmission	
66.118108	1.1	884	=-0.0459* $C2+45.42	
128.639216	2.1	883	4.9	