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Identification of novel regulators of plant transpiration by large-scale thermal imaging screening in Helianthus annuus.

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Dear Editor,

We would like to submit the original protocol presented in our manuscript titled "**Identification of novel regulators of plant transpiration by large-scale thermal imaging screening in *Helianthus annuus*.**" for consideration for publication in ***JoVE***.

Problem

Plant adaptation to biotic and abiotic stresses is governed by a variety of factors, in particular the regulation of stomatal aperture in response to water deficit or pathogens. Identifying small molecules that regulate stomatal movement can therefore contribute to understanding the physiological basis by which plants adapt to their environment. Large-scale screening approaches that have been used to identify regulators of stomatal movement have potential limitations: some rely heavily on the abscisic acid (ABA) hormone signaling pathway, therefore excluding ABA-independent mechanisms, while others rely on the observation of indirect, long-term physiological effects such as plant growth and development.

Additionally, while there are many ways to treat plants with bioactive molecules, most of them are not well suited for a large-scale study of stomatal movement.

Solutions offered by the protocol and significance

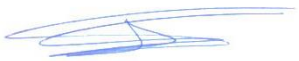
Here, we present a large-scale method to identify new compounds regulating plant transpiration that does not necessarily involve ABA- or known drought-responsive mechanisms and allows for efficient and reliable treatment of plants. In this system, *Helianthus annuus* plants are treated using a root feeding approach that consists of cutting the primary root of seedlings grown hydroponically and dipping the cut site into the sample solution. Once treated, the effect of each compound on the transpiration of plants is measured using an infrared thermal imaging camera. The relative change in foliar temperature following chemical treatment thus provides a direct mean to quantify the plant transpiration.

While the protocol in this manuscript is applied to seedlings of the sunflower *Helianthus annuus*, it is versatile in regard to the plant species that can be tested, the nature of the library of compounds and the type of imaging (thermal or chlorophyll fluorescence for example).

To our knowledge, only one chemical screening focused on stomatal movement has been published in August 2018 by the laboratory of Dr. Kinoshita from Nagoya University (Toh et al., 2018). The strategy developed by Toh et al. consisted in three sequential rounds of screens to assess the stomatal aperture in *C. Benghalensis* leaf disks immerged in the compounds. Only the last round included a quantitative evaluation of the effect. In contrast, our approach solely generates quantitative data, and differences are directly assessed by statistical treatment. Moreover, data are collected by the thermal imaging system every five minutes during the incubation, allowing us to (i) take into account natural discrepancies in stomatal aperture between plants and to (ii) perform quantitative analysis of the response over time.

We believe that this protocol will be useful to effectively identify molecules able to trigger stomatal closure or promote stomatal opening, which has major implications for understanding the signals that regulate stomatal conductance and plant adaptation to environmental stresses.

This manuscript has not been submitted for publication elsewhere. We confirm that the manuscript has been read and approved by all authors. Thank you for your consideration of our manuscript. We look forward to your review.



Sincerely,

Fabien Jammes
Assistant Professor of Biology
Pomona College

TITLE:

Identification of Novel Regulators of Plant Transpiration by Large-Scale Thermal Imaging Screening in *Helianthus annuus*

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KEYWORDS:

Helianthus annuus, transpiration, stomatal movement, thermal imaging, screening, chemical library

SUMMARY:

We provide a method for identifying modulators of foliar transpiration by large-scale screening of a compound library.

ABSTRACT:

Plant adaptation to biotic and abiotic stresses is governed by a variety of factors, among which the regulation of stomatal aperture in response to water deficit or pathogens plays a crucial role. Identifying small molecules that regulate stomatal movement can therefore contribute to understanding the physiological basis by which plants adapt to their environment. Large-scale screening approaches that have been used to identify regulators of stomatal movement have potential limitations: some rely heavily on the abscisic acid (ABA) hormone signaling pathway, therefore excluding ABA-independent mechanisms, while others rely on the observation of indirect, long-term physiological effects such as plant growth and development. The screening method presented here allows the large-scale treatment of plants with a library of chemicals coupled with a direct quantification of their transpiration by thermal imaging. Since evaporation of water through transpiration results in leaf surface cooling, thermal imaging provides a non-invasive approach to investigate changes in stomatal conductance over time. In this protocol, *Helianthus annuus* seedlings are grown hydroponically and then treated by root feeding, in which the primary root is cut and dipped into the chemical being tested. Thermal imaging followed by

statistical analysis of cotyledonary temperature changes over time allows for the identification of bioactive molecules modulating stomatal aperture. Our proof-of-concept experiments demonstrate that a chemical can be carried from the cut root to the cotyledon of the sunflower seedling within 10 minutes. In addition, when plants are treated with ABA as a positive control, an increase in leaf surface temperature can be detected within minutes. Our method thus allows the efficient and rapid identification of novel molecules regulating stomatal aperture.

INTRODUCTION:

Stress tolerance in plants is a polygenic trait influenced by a variety of molecular, cellular, developmental and physiological features and mechanisms[1]. Plants in a fluctuating environment need to continuously modulate their stomatal movements to balance the photosynthetic demand for carbon while maintaining sufficient water and preventing pathogen invasion[2]; however, the mechanisms by which these trade-off "decisions" are made are poorly understood[3]. Introducing bioactive molecules into plants can modulate their physiology and help in probing new mechanisms of regulation.

The large-scale screening of small molecules is an effective strategy used in anti-cancer drug discovery and pharmacological assays to test the physiological effects of hundreds to thousands of molecules in a short period of time[4,5]. In plant biology, high-throughput screening has showed its effectiveness for example in the identification of the synthetic molecule pyrabactin[6], as well as the discovery of the long-sought receptor of abscisic acid (ABA)[7,8]. Since then, agonists and antagonists of ABA receptors, and small molecules able to modulate the expression of ABA-inducible reporter genes have been identified[9-15]. High-throughput screening approaches currently available to identify small compounds that can modulate stomatal aperture have some drawbacks: (i) protocols revolving around the ABA signaling pathway may prevent the identification of novel ABA-independent mechanisms, and (ii) in vivo strategies used for the identification of bioactive small molecules rely primarily on their physiological effects on seed germination or seedling growth, and not on the regulation of plant transpiration per se.

Additionally, while there are many ways to treat plants with bioactive molecules, most of them are not well suited for a large-scale study of stomatal movement. Briefly, the three most common techniques are foliar application by spraying or dipping, treatment of the root system, and root irrigation. Foliar application is not compatible with the most common and rapid methodologies to measure stomatal aperture since the presence of droplets at the leaf surface interfere with large-scale data collection. The major limitations of root irrigation are the large sample volume requirements, the potential retention of the compounds by elements in the rhizosphere, and the reliance on active root uptake.

Here, we present a large-scale method to identify new compounds regulating plant transpiration that does not necessarily involve ABA- or known drought-responsive mechanisms and allows for efficient and reliable treatment of plants. In this system, *Helianthus annuus* plants are treated using a root feeding approach that consists of cutting the primary root of seedlings grown hydroponically and dipping the cut site into the sample solution. Once treated, the effect of each compound on the transpiration of plants is measured using an infrared thermal imaging camera.

Since a major determinant of leaf surface temperature is the rate of evaporation from the leaf, thermal imaging data can be directly correlated to stomatal conductance. The relative change in foliar temperature following chemical treatment thus provides a direct means to quantify the plant transpiration.

H. annuus is one of the five largest oilseed crops in the world[16] and discoveries made directly on this plant may facilitate future transfers of technology. In addition, *H. annuus* seedlings have large and flat cotyledons, as well a thick primary root, which was ideal for the development of this protocol. However, this method can be readily adapted to other plants and a variety of compounds.

This protocol can be used to effectively identify molecules able to trigger stomatal closure or promote stomatal opening, which has major implications for understanding the signals that regulate stomatal conductance and plant adaptation to environmental stresses.

PROTOCOL:

1. Growing the plants

1.1. Add a 4 cm-thick layer of fine vermiculite to standard 10 in. x 20 in. (254 mm x 501 mm) plant trays with no holes.

1.2. Place the seed holders (see **Table of Materials**) 2 cm apart in the plant trays.

1.3. Fill seed holders with vermiculite.

1.4. Place a sunflower seed with its pointed end down in each seed holder, pushing down so half of the seed remains exposed.

NOTE: A sunflower seed is asymmetrical and the pointed end from where the radicle will emerge should point downwards. Proper seed placement is important as the reorientation of root and stem are not possible within the seed holders. The rounded end of the seed should extend past the top of the seed holder.

1.5. Once the seeds are in place, cover them with an additional 2 cm-thick layer of fine vermiculite. Water by misting from above. The surface should remain wet after an hour. If that is the case, cover the trays with lids.

1.6. Grow plants in a growth chamber or a greenhouse. Recommended conditions are a light intensity of 140 µmol photons·m^{-2}·s^{-1} and a photoperiod of 16 h light at 22 °C and 9 h dark at 20 °C for 5 days.

NOTE: Watering should not be necessary unless the surface becomes visibly dry.

2. Set up of the hydroponic system

2.1. Find an appropriately sized container suitable for growing plants hydroponically. The size of the container should be adapted to the space available in the growth chamber or greenhouse. A minimal depth of 15 cm is recommended.

2.2. Fill the container with distilled water and add general hydroponics fertilizer as indicated by the manufacturer. The resulting hydroponic solution should be aerated and in constant movement, which can be achieved by using air and water pumps.

2.3. Prepare the hydroponics floaters.

2.3.1. Cut a sheet of 2 cm-thick expanded polystyrene foam (see **Table of Materials**) to the dimensions of the container. The sheet should cover most of the surface of the container in order to limit the growth of algae. A wood burning tool is effective for cutting polystyrene foam and is versatile enough for this protocol.

CAUTION: Fumes or vapor released during hot cutting of polystyrene foam are serious health hazards. Use proper respiratory protection. Users can also satisfy ventilation requirements by cutting the foam under a fume hood.

2.3.2. Make holes (1–2 cm in diameter) in the polystyrene foam sheet using a wood burning tool. The distance between the centers of two holes can be adjusted to the needs of the experiment. However, a minimal distance of 2.5 cm is recommended.

3. Transfer of seedlings to hydroponics and plant growth

3.1. Gently pull out 5-day old seedlings from the vermiculite and transfer immediately to a container filled with water for 30 min. This step will remove excess vermiculite and soften the remaining pericarps. The emerging primary root should be visible.

3.2. Remove the pericarp walls by hand if needed in order to optimize the future expansion of the cotyledons.

3.3. Transfer the seedlings within the seed holders to the polystyrene foam floater. Grow the plants with a light intensity of 140 µmol photons·m^{-2}·s^{-1}, a relative humidity of 65% and a photoperiod of 16 h light at 22 °C and 9 h dark at 20 °C for 2 days.

4. Preparation prior to treatment

NOTE: This procedure is for testing 20 chemicals from a small compound library in triplicate, with 100 µM ABA in 10 mM MES-KOH (pH = 6.2) and 10 mM MES-KOH (pH = 6.2) containing 1% (v/v) dimethyl sulfoxide (DMSO) as positive and negative controls, respectively.

4.1. Ensure that there are enough plants ready for treatment. Plants ready for treatment should be mature enough to have fully unfolded cotyledons, but young enough that lateral root proliferation is minimal. To do a standard screening, 69 such plants are needed.

4.2. Remove the 96-well plate containing the small compounds from the -80 °C freezer. Thaw at room temperature.

4.3. Prepare 80 mL of 10 mM MES-KOH buffer adjusted to a pH of 6.2 with 1 M KOH.

4.4. Label cap-less 2 mL microtubes. Prepare at least six tubes for the negative control treatment (10 mM MES-KOH (pH = 6.2) containing 1% (v/v) DMSO). Use three tubes for ABA treatment (100 µM ABA in 10 mM MES-KOH pH = 6.2), positive control). Use the remaining 60 tubes to analyze the effect of the 20 chemicals in triplicate.

4.5. Transfer 10 µL of each chemical (10 mM in DMSO) into each of the three appropriately labeled tubes. Pipet 10 µL of 10 mM ABA dissolved in DMSO into three tubes and 10 µL of DMSO into the six control tubes.

CAUTION: By nature, some compounds may cause serious health effects and users must take appropriate protective measures.

4.6. Add 990 µL of 10 mM MES-KOH (pH = 6.2) to each of the 69 tubes. Dispense the MES buffer with enough force to mix the chemical with the MES buffer but be very careful not to use so much force that the chemicals and MES buffer squirt out of the tube. Alternatively, vortex at low speed.

5. Set up the thermal imaging camera

5.1. Mount the thermal imaging camera on a copy stand. Connect all cables to a laptop.

NOTE: The recording is done under conditions of temperature (20 °C to 25 °C), humidity (50% to 70%) and light quality (110 to 140 µmol photons·m^{-2}·s^{-1}) similar to those used to grow the plants.

5.2. Turn on the camera then the laptop and open the thermal imaging analysis software.

NOTE: The subsequent instructions for recording apply to a specific software used (see **Table of Materials**).

5.3. Adjust the recording settings.

5.3.1. Mouse over the **record** red button at the top of the central window. A dropdown menu will appear. Click on the wrench icon **Record Settings**.

5.3.2. Select the appropriate record mode and options. The **record periodically** option, with one frame captured per minute and a manual stop could be used. Note the file destination where the

software will save the video. Close the **Record Settings** window.

6. Plant preparation and treatment

6.1. Place the cap-less tubes containing the chemicals in tube racks. Alternatively, a polystyrene foam sheet can be cut and poked with a wood burning tool as described in step 2.3 to make a custom tube rack. The diameter of each hole should be very close to the external diameter of the capless tubes in order to hold them firmly.

NOTE: The field of view of the camera is a limiting factor that should be taken into consideration when deciding on how the cap-less tubes will be held in place.

6.2. Evenly distribute the positive and negative control tubes as well as the experimental tubes in the racks to account for position-related bias[17].

6.3. Prepare the following materials next to the plants grown hydroponically: microdissection scissors, a shallow dish with water, delicate task wipes, the 69 cap-less tubes containing the different chemicals.

6.4. Repeat the following steps for each plant to be treated. The sunflower sprout will always remain in the seed holder.

6.4.1. Carefully lift the seed holder and rapidly dip the root into the shallow dish containing water.

6.4.2. Cut the primary root underwater to prevent cavitation. The cut should occur 0.8–1 cm underneath the most basal end of the seed holder.

6.4.3. Inset the freshly cut plant into one of the tubes containing the chemicals.

6.4.4. If there are any drops of water on the cotyledons, gently dab them dry with a delicate task wipe.

NOTE: These four steps must be done as quickly as possible (10 min or less) to prevent inconsistencies in the kinetics study.

6.5. Move the plants under the thermal imaging camera and ensure that all plants are within the field of view of the camera. Adjust camera height and racks position as necessary.

7. Recording

7.1. Focus the camera to the surface of the cotyledons by pressing **Ctrl** + **Alt** + **A**.

7.2. Mouse over the red button and click on the **Record a Movie** option. A new window

confirming the recording should open.

7.3. Stop the recording 1–2 hours later.

NOTE: The protocol can be paused here.

8. Data collection

8.1. Go to **File | Open | Browse** for the correct .SEQ file and open it.

8.2. Stop playing the movie.

8.3. On the left side of the main window, click on **add a measurement cursor ROI (3x3 pixels)** icon. ROI stands for Region of Interest.

8.4. Mouse over the center of a cotyledon of the first plant and left-click once. The cursor 1 is now in place. Label the second cotyledon of the first plant by repeating the procedure. The order of the labels should be noted.

8.5. Repeat the procedure. All plants should be labeled with two cursors.

8.6. Click on the **Edit ROIs** icon. In the main window, left click and hold on the top left corner and scroll to the bottom right corner to select all the ROIs.

8.7. Mouse over the **Statistic Viewer** icon and select **Temporal Plot**. A new window will open.

8.8. Run the movie. A graph will fill with the data.

8.9. In this window, click on the double arrow in the top right corner to open a new menu.

8.10. Click on the **Save** icon. Save as **X and Y values in the plot (.csv)**. Close the software once data has been exported.

9. Data analysis

9.1. Open the .csv file using a data analysis software (e.g., Microsoft Excel). Note that the three first columns (A to C) provide information about the frame number, the absolute time and the relative time. The remaining columns give the temperature of each ROI over time.

9.2. Decide on the nature of the statistical tool to be used; this decision depends on different factors including the experimental design.

NOTE: In our example, a standard score, or z-score, is calculated for each sample based on population mean and population standard deviation. For each sample, a p-value is then

calculated from the z-score. This method allows the confirmation of the positive and negative controls as well as the identification of new compounds to be tested further.

REPRESENTATIVE RESULTS:

An experiment using the red dye Erythrosine B (0.8 kDa) demonstrates the ability of chemicals to be visibly absorbed through a cut root into the cotyledons of a sunflower seedling within 10 minutes (**Figure 1**).

When plants are treated with ABA, an increase in leaf temperature is detected in sunflower cotyledons within minutes. This increase in leaf temperature is associated with a decrease in stomatal aperture and stomatal conductance. Increased foliar temperature is observed 15 min after treatment with 10 µM ABA (p-value = 0.02) and 20 min with 5 µM ABA (p-value = 0.003) (**Figure 2**). Overall, these results show that measurements of leaf temperature by thermal imaging is a good proxy for measuring stomatal aperture and conductance.

Figure 3 shows a proof of concept experiment using a subset of 20 chemicals from the NatProd Collection with positive (100 µM ABA) and negative controls. In this representative experiment, a standard-score-based statistical treatment allows the identification of chemicals promoting stomatal closure or, while the assay should be optimized for that specific purpose, chemicals promoting stomatal opening. In the given example, a heat-map visualization of the standard scores allows the rapid identification of chemicals #02 and #16 as potential candidates.
Figure 4 summarizes the important steps of the workflow.

FIGURE LEGENDS:

Figure 1. Effectiveness of the cut root feeding approach. (**A**) A seedling fed for 1 h with Erythrosine B in 10 mM MES-KOH (pH = 6.2) is visibly red (right image) compared to the control (left image). The images were taken after the cut root feeding followed by an overnight incubation in absolute ethanol to remove the natural plant pigments. Bar = 10 mm. (**B**) Accumulation of Erythrosine B in cotyledons over time. Erythrosine B can be detected by spectrophotometry in plant extracts from cotyledons 8 min (p-value=0.032) after the transfer of cut-root sunflower seedlings to the dye. Error bars indicate SEM. * indicates p-value < 0.05 (n = 3).

Figure 2. Relationships between leaf temperature, stomatal aperture and conductance to show sensitivity of the experimental design. (**A**) Representative image showing differences in leaf temperature between 100 µM ABA-treated (+ABA) and non-treated (Control) sunflower seedlings after 30 min visualized by thermal imaging. (**B**) Left: plants treated with 100 µM ABA for 30 min show an increased temperature compared to control plants (* indicates p-value < 0.01), n = 3). Right: measurements of stomatal aperture on epidermal peels from the same plants show a decrease in stomatal aperture (width/length) (* indicates p-value < 0.01, n = 3, number of stomata per plant ≈ 162). (**C**) Leaf conductance measured with a leaf porometer and coupled with leaf temperature measurements show that there is a strong correlation (Pearson's coefficient = -0.89, n = 6) between leaf surface temperature and stomatal conductance. Plants

treated with 100 µM ABA for 30 min show an increased temperature and decreased conductance compared to control plants (n = 6). (**D**) Dose-response study shows reduced leaf temperature in plants treated with ABA concentrations as low as 5 µM after 20 min of treatment (p-value = 0.0037, n = 3). Error bars indicate SEM.

Figure 3. Representative results from the screening of 20 chemical compounds. (**A**) Heatmap of Z-scores reflecting plant responses to 20 compounds tested in triplicates. Dark red and dark blue indicate confidence level of >99% for stomatal closure and opening, respectively. Six plants were treated with DMSO (control), three were treated with 100 µM ABA, and other plants were treated with 100 µM of chemical in triplicate. Plants responding to compound 16 (C#16) show a stomatal closure similar to that observed in ABA-treated plants. Two plants out of three treated with compound 02 (C#02) show a significant increase in stomatal opening. (**B**) Kinetics of the response of plants to compounds 02 and 16. Average changes in temperature over time are shown for plants responding to control treatment (n = 6), 100 µM ABA (n = 3) or 100 µM of each compound (n = 3). Error bars indicate SEM. Changes in temperature are consistently statistically significant after 10 min of ABA treatment (p-value = 0.026, n = 3), 15 min of treatment with C#16 (p-value = 0.030, n = 3) and 71 min of C#02 (p-value = 0.044, n = 3) compared to control. Fluctuations shared by all the samples is background noise due to the dynamic control of ambient temperature in the growth chamber.

Figure 4. Summary of the screening workflow. Note that the images represent important steps and are independent from each other.

DISCUSSION:

The number of compounds that can be tested on a given day mostly depends on (i) the environmentally controlled space available to grow the plants and to perform the screen, as well as (ii) the number of individuals who can be involved in step 6 of the protocol. We recommend the use of three experimental replicates to consolidate the interpretation of the results after statistical treatment. In a typical day, one to two individuals can screen 60 compounds in triplicates without difficulty by testing for example [60 chemicals + 6 negative (DMSO) controls + 3 positive (ABA) controls] in the morning, midday and afternoon.

This method relies on healthy seedlings with fully developed cotyledons. As the imaging occurs from above, an ideal seedling should show an angle of 90° between the hypocotyl and the cotyledonary blade in order to collect as much information as possible. This angle is mainly regulated by light and should therefore be optimized by adjusting the growing conditions. Our results show that it takes around 10 min for a chemical to reach the cotyledons and a few more minutes to respond to a chemical such as ABA. This observation makes step 6.4 the most time-sensitive step in the protocol. It is therefore critical to treat all the plants in a given assay in less than 15 min to avoid discrepancies between the plant responses. Among the external factors that passively affect foliar temperature measurements, ventilation is likely to introduce position-related biases or significant variability among replicates. Users should exercise caution by controlling ventilation flows and limit position-related biases by randomly distributing the

samples before recording. To account for other potential factors, recording should be done under similar temperature, humidity, and light conditions to those used to grow the plants since any changes in these conditions may affect stomata closure and/or foliar temperature. Finally, a compound able to modulate stomatal closure should be evaluated for its toxicity. This holds particularly true if the compound triggers stomatal closure, as it is known to be an indirect consequence of intense stress experienced by the plant.

By providing an effective delivery method of bioactive molecules and a method to directly measure plant transpiration, this protocol addresses some of the drawbacks associated with current screening approaches, as mentioned in the introduction. Our protocol is not exclusive to sunflower seedlings and can be applied to most dicots with a hypocotyl to cotyledon angle of 90°. Thermal imaging of *Arabidopsis* cotyledons is effective[18,19] and our protocol could therefore be adapted to seedlings with similarly small cotyledons. In addition, chlorophyll fluorescence imaging could be used to measure photosynthetic performance in combination. While less time-effective, measurements of the transpiration-driven accumulation in cotyledons of Erythrosine B added to each chemical could potentially be used to evaluate transpiration rates if a thermal imaging camera is not available. In all, this large-scale screening method efficiently evaluates plant foliar response to bioactive molecules and is readily adaptable to a variety of applications.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

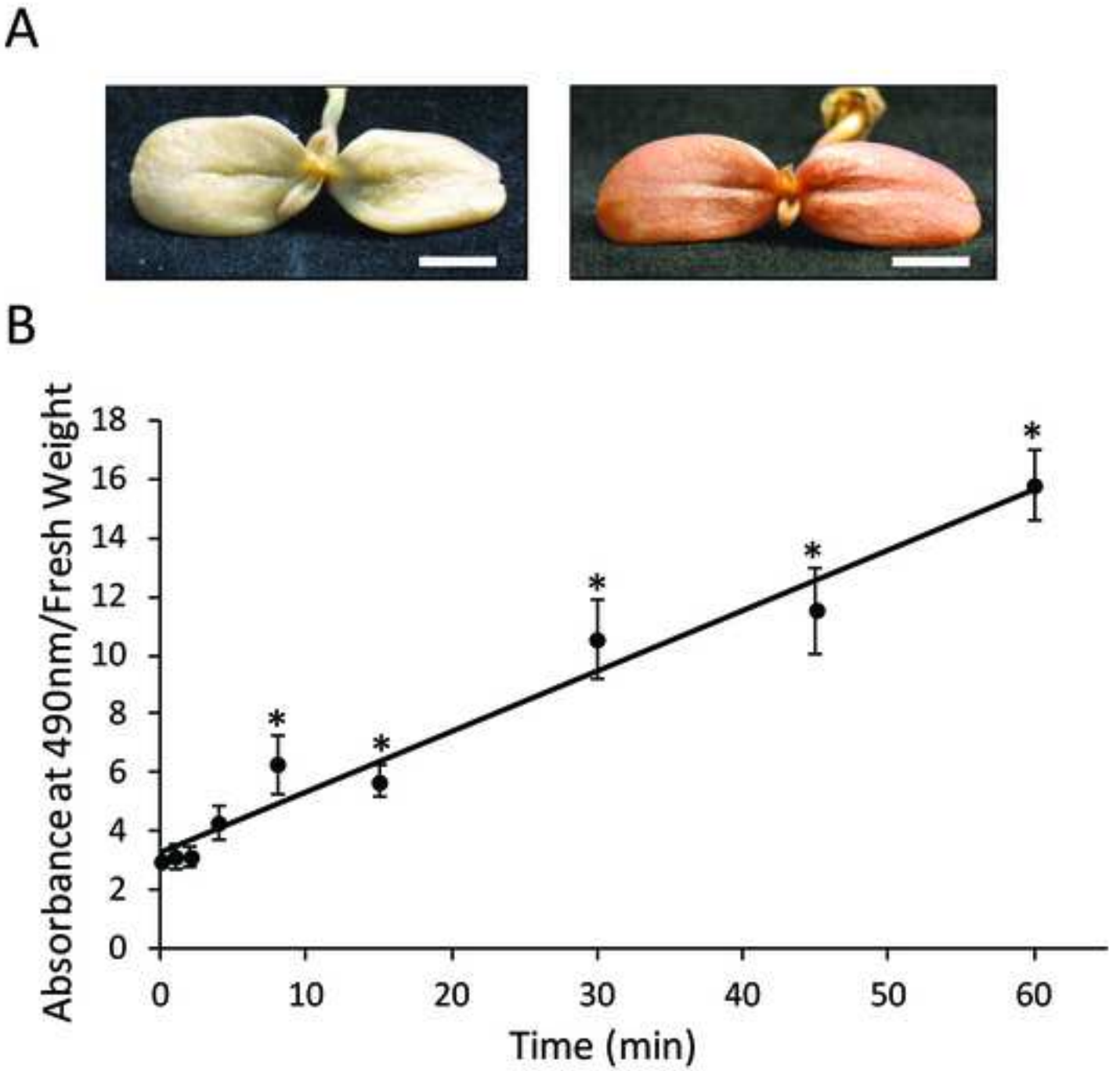

Figure 2

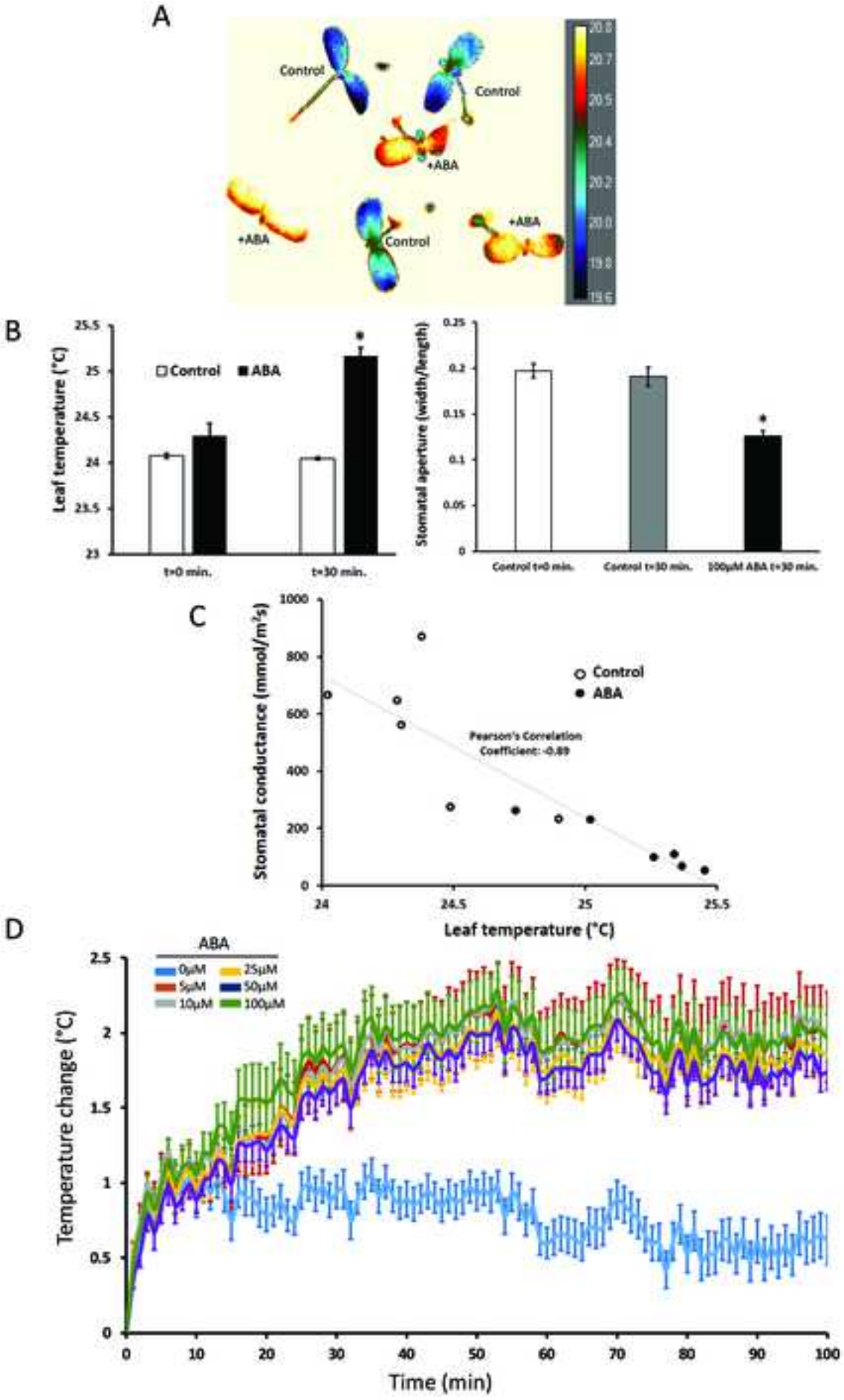

Figure 3

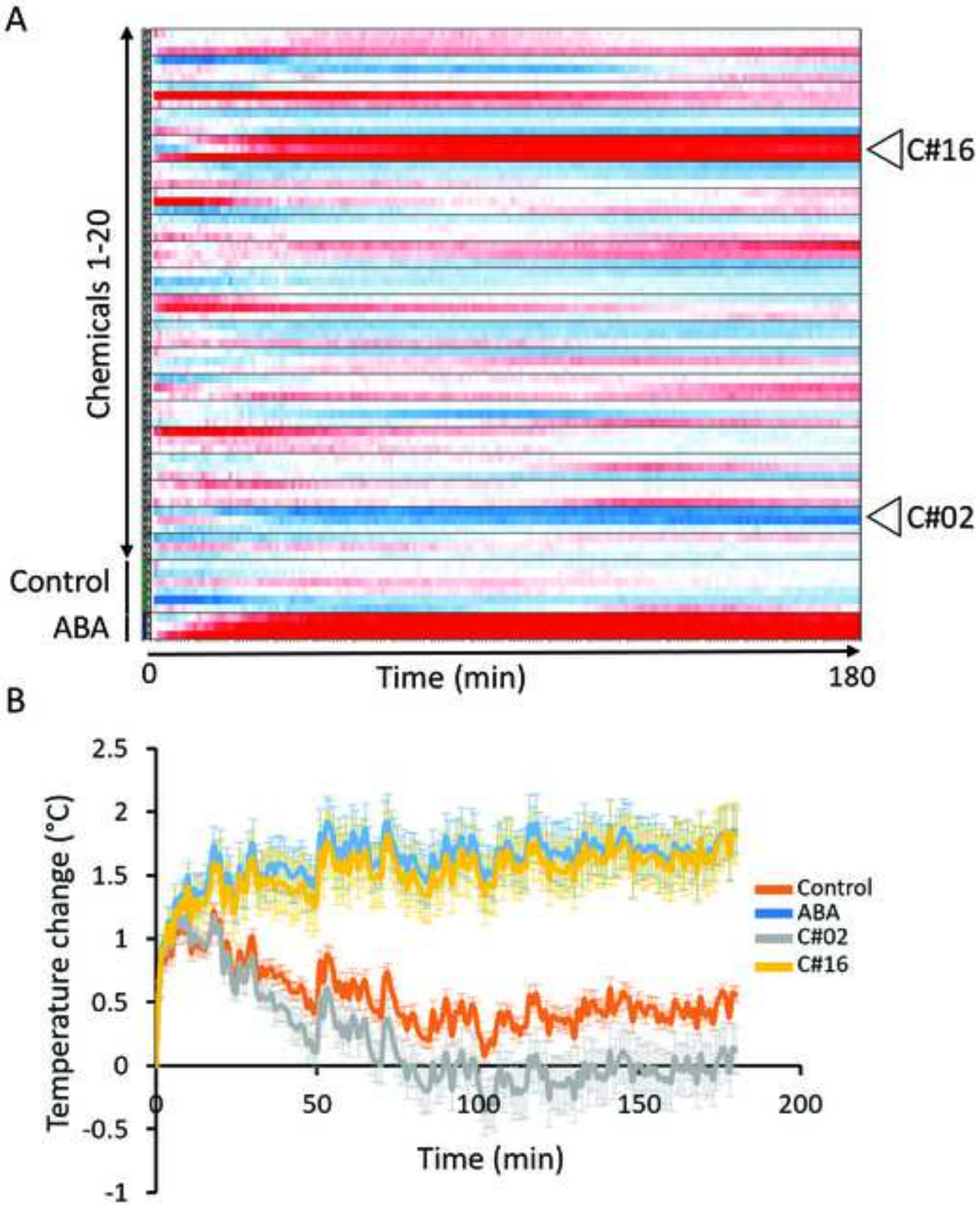

Figure 4

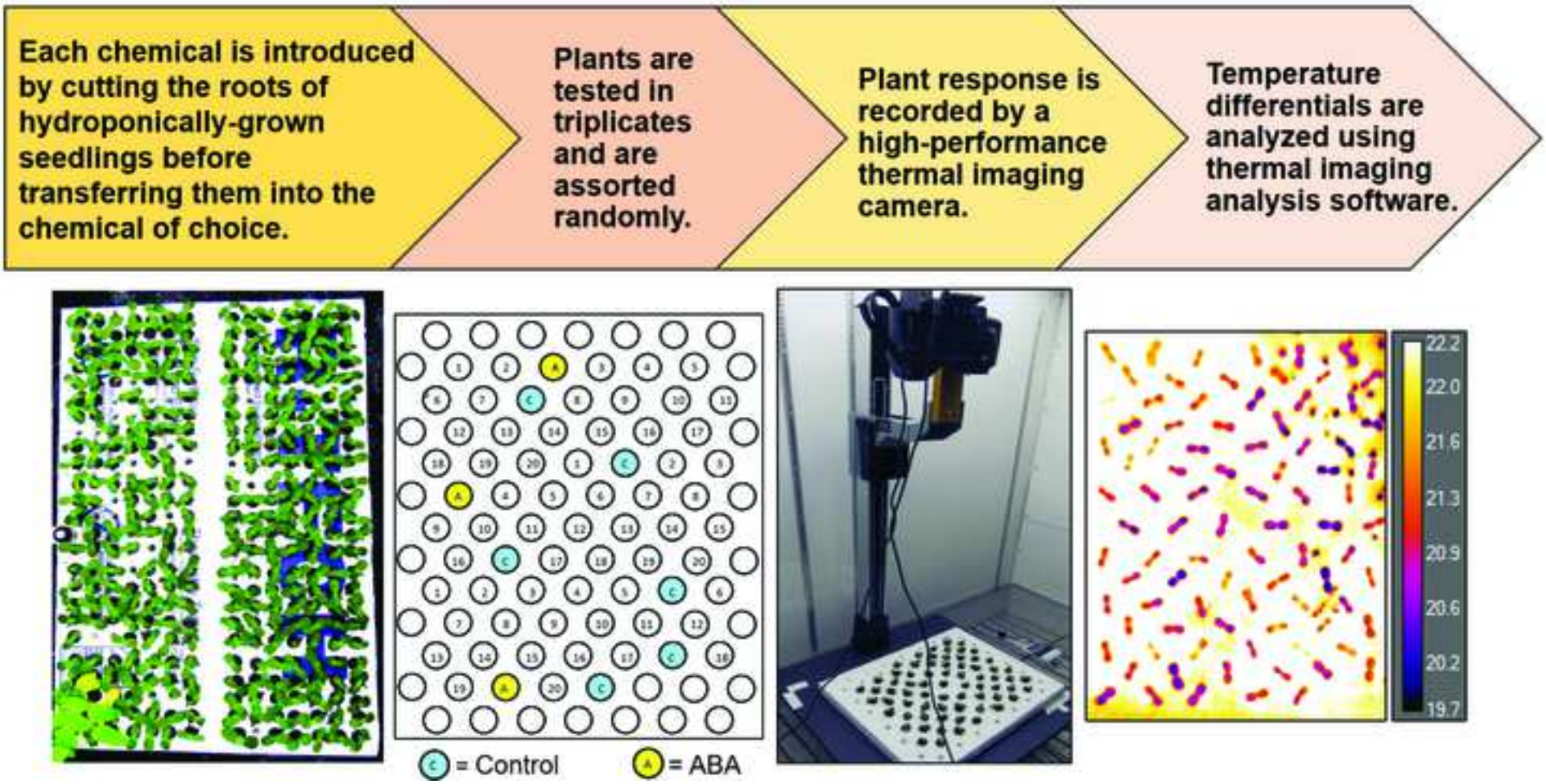

Table of Materials

Click here to access/download;Table of Materials;Guo et al_Table_of_Materials_Revised.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1020 plastic growing trays without drain holes			Standard 10 x 20 inch trays
2.0 mL microtubes, capless	Genesee Scientific	22-283NC	
Abscisic acid (ABA)	Sigma-Aldrich	A1049	
Air pump	Active Aqua	AAPA7.8L	2 Outlets, 3W, 7.8 L/min
Airstones			
Chemical compound library	MicroSource Discovery		Natural Product Collection
Creative Versa-Tool (wood burning tool)	Nasco	9724549	
Dimethylsulfoxide (DMSO), plant cell culture tested	Sigma-Aldrich	D4540	
Dwarf Sunspot Sunflower seeds	Outsidepride.com		
Erythrosin B	Sigma-Aldrich	200964	
Hydroponics fertilizer set (FloraBloom, FloraGrow, FloraMicro)	General Hydroponics	GL51GH1421.31.11	
Kimwipes Delicate Task Wipers	Kimberly-Clark Professional	34155	
Laptop	Dell		
MES hydrate	Sigma-Aldrich	M2933	
Microdissection scissors			
Microsoft Excel	Microsoft		
Potassium hydroxide (KOH)	Sigma-Aldrich	P5958	
ResearchIR Software	FLIR		
R-Tech Rigid Polystyrene Foam Board	Insulfoam		
Seedholders	Araponics	N/A	
Super Tub (plastic utility tub)	Maccourt	ST3608	36 x 24 x 8 inch tub used for hydroponics
T450sc LWIR (Long-Wave Infrared) Handheld Thermal Imaging Camera	FLIR	FLIR-T62101	Comes with required charging cable and USB cab

Vermiculite		
Water filter	SunSun	HW-304B Pro Canister Filter

ole needed to connect to laptop

Response to the Editor and Reviewers.

Dear Dr. DSouza,

Thank you so much for handling our manuscript. We have now addressed all points raised by the reviewers. In the process, we have added substantial new data including (1) the measurements of stomatal apertures and (2) the measurements of stomatal conductance that were requested by Reviewer #2.

We feel that our efforts have consolidated our manuscript. Below is a point-by-point rebuttal to all comments raised in the email received. We hope you will find that our paper is now suitable for publication in JOVE.

Thank you very much for considering our work.

Sincerely,

Fabien Jammes

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

- We have proofread the manuscript.

• Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in the imperative voice:2.1, 4.1, 6.1, 9.3etc

- The language has been modified as requested.

• Protocol Detail:
1) 1.1: What is the size of each plant tray?

- The size of the tray is now indicated.

• Protocol Numbering: Please add a one-line space after each protocol step.

- One-line spaces after each protocol step have been added.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We think that our manuscript follows the above guidelines.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

- We made some changes and believe that our discussion fits with the focus of the journal.

• References:
1) Please edit your references to comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

- We have edited the references

2) Please spell out journal names.

- Journal names are now spelled out.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Styrofoam, FLIR

- The word "Styrofoam" has been replaced with the words "polystyrene foam".

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

-Company-specific language does not appear in the manuscript anymore.

2) Check fig 4 as well

-Mention of the "ResearchIR software" has been removed from Figure 4. Note: the last two steps in the workflow presented in Figure 4 were not in the right order. This has now been corrected.

• Table of Materials:

1) Please sort the table by alphabetical order.

- The Table of Materials is divided into sections and we have alphabetized the components within each section.

• Please define all abbreviations at first use.

-All abbreviations have been defined.

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NOTE: A new author has been added to the list. Figure 1 has been revised to fix a typo (the word "weight" was misspelled).

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript presents a rapid screening method on the chemicals other than ABA that could regulate stomatal aperture, with a direct quantification of transpiration by thermal imaging. Among 20 chemicals tested, some chemicals result in stomatal closure, particularly C#16 is similar to ABA and effectively induces stomatal closure, and some promote stomatal opening, particularly C#2. Identification of these novel chemicals provides new aspects for the research on the stomatal regulation mechanisms. The experimental protocol is clearly described.

Major Concerns:

May add some discussion on the possible mechanisms of regulating stomatal aperture by these molecules.

We appreciate the interest of Reviewer #1 in the preliminary results regarding C#16 and C#2. However, we are conflicted between our need to further confirm and investigate these compounds and the scope of this method journal. Overall, we believe that, with the addition of the new experiments requested by Reviewer #2, we are providing a robust experimental approach in agreement with the goals of the journal. Regarding the discussion, we have received the specific comment from the Editor *"Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol."* Therefore, we have decided to focus our discussion on the technique and we hope Reviewer #1 will understand our position.

Reviewer #2:

This manuscript describes a method in which small soluble molecules can be hydroponically fed in sunflower seedlings, and stomatal responses can be indirectly detected by changes in the canopy leaf temperature using infra-red thermal imaging. In the proposed method, roots are first cut and dipped into the chemical. After a few minutes, cotyledon temperature changes are evaluated. The method is proposed as a suitable approach for screening molecules that control stomatal movements. Although the general idea seems like a good idea, major revision of this manuscript would be necessary before it can be considered for JoVE:

Abstract

In the following sentence (line 45): "… plants treated with ABA as a positive control, stomatal closure can be detected…" The authors did not show data on stomatal aperture measurements. The hypothesis is that the cotyledons are warmer due to stomatal closure, but this was not truly tested.

As suggested by the reviewer, new experiments 2B and 2C show that the increase in temperature of the cotyledons is accompanied by (1) a decrease in stomatal aperture and (2) a decrease in stomatal conductance.

While this may not be considered necessary anymore, we have edited the sentence to *"… plants are treated with ABA as a positive control, an increase in leaf surface temperature can be detected within minutes*".

Representative results

1- In the proof of concept in Figure 1 - Seedlings were pre-incubated in ethanol (concentration is not provided) overnight. This treatment alone could cripple the plants and the stomata, depending on the concentration and the length of the "overnight" treatment. The method by which this entire figure was made was not mentioned in the protocol.

We would like to thank the reviewer for giving us the opportunity to improve our manuscript by clarifying the methods used to generate the representative results. In response to the reviewer's comment, we have revised the legend for Figure 1. Briefly, the plants had been fed for an hour with the red dye in 10mM MES-KOH and then fixed/distained overnight to remove chlorophyll pigments.

2- Figure 3B - It seems quite unlikely that two distinct chemicals in distinct seedlings would show an almost identical temperature change response including all the fluctuations. This might be indicative of another (not considered) input affecting leaf temperature.

We thank the reviewer for this comment. We believe the following points provide clarification:
1. Each line in Figure 3B was generated by averaging measurements from at least three independent plants (the measurements for each independent plant can be seen in Figure 3A).
2. Stomatal closure/opening will rapidly lead to a plateau of the surface temperature, i.e. the temperature cannot increase/decrease indefinitely once all stomata are closed/open.
3. The fluctuations are shared by *all* curves, and correspond to background noise due to the dynamic control of the ambient temperature within the growth chamber (the chamber maintains a defined average temperature by constantly warming up or cooling down the environment). Subtracting the background noise could have smoothened the lines but we preferred not to manipulate the data too extensively.

We have added the following sentence in Figure 3B legend "Fluctuations shared by all the samples is background noise due to the dynamic control of ambient temperature in the growth chamber."

Protocol
1- Relative humidity of the growth conditions was not mentioned, but this is crucial in such experiments as it directly affects stomatal apertures and stomatal development.
Step 3.3 and the note to step 5.1 have been revised according to your request.

2- The authors use DMSO as the solvent in their experiments. It is known that DMSO has severe effects in plants. The authors should try diluting ABA in ETOH instead, which seems to be less toxic to plants.
We believe the note below step 4 in the protocol was confusing and it has been edited for clarification. Step 4.4 has also been edited for the same reason.
DMSO or EtOH to dissolve and use ABA in plants are commonly used in the literature. Both can be toxic to the plants if inappropriate concentrations are used. While we classically use EtOH in our laboratory, we opted for DMSO because it is the standard solvent used in compound libraries (see https://dev.biologists.org/content/144/3/499 for reference)
In all of our control experiments, the control plants have received the same concentration of DMSO (1%) as the plants treated with ABA and no adverse effect prevented us to conduct our experiments.

3- Experiments to be considered before publication: A- Check stomatal apertures in the seedlings in the same time frame.
We have performed the suggested experiment and the new Figure 2B shows that ABA treatment by cut-root feeding directly causes stomatal closure.
B- If possible, the more reliable stomatal conductance measurements would be an important addition to this work as a proof of concept.
We have performed the suggested experiment and the new Figure 2C shows a strong correlation between leaf temperature and stomatal conductance. Figure 2C also shows that ABA treatment by cut-root feeding causes a decrease in stomatal conductance. Overall, these results show that measurements of leaf temperature by thermal imaging is a good proxy for measuring stomatal aperture and conductance.
C-Check the cotyledon temperature before and after the whole manipulation (including cut) over a period of a few hours. Does the manipulation alone affect leaf temperature?
We think the suggested experiment goes beyond our goal that is to measure foliar temperature over a period of maximum one hour for the screening of a compound library (note that figure 3 shows data over 3 hours). Additionally, since each plant is manipulated in the same fashion, any effect on leaf temperature from the manipulation would apply to all plants and thus be controlled.

4- Figure 4 - Some of the ABA-treated seedlings are clearly colder than the control?

Our intention with Figure 4 is only to provide a visual summary of the workflow. These images are not related to each other (number/position of the plants are different). We have added the following sentence to Figure 4 legend to avoid any confusion: "*Note that the images represent important steps and are independent from each other.*"

Figure legends

1- In figure 2 the legend is inaccurate. Leaf transpiration was not evaluated in this assay, but changes in leaf temperature.

The word "transpiration" in the legends for Figure 2A and Figure 2D (2B in the initial manuscript) has been replaced by "leaf temperature". Similar changes have been made throughout the manuscript.

2- The same applies for figure 2B. The authors did not measure stomatal conductance and the experiment is simply reporting changes in leaf temperature. Gas exchange evaluations are needed to assess this method.

We have performed two additional experiments presented in Figure 2B and Figure 2C showing evidence for the relationship between leaf surface temperature, stomatal aperture and stomatal conductance.

The paragraph describing Figure 2 in the "Representative Results" has been changed to integrate the new experiments.

We are grateful for the constructive comments that have helped strengthen our data and hope the reviewers will recognize our best effort to answer them.

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Author(s):	Konnie Guo, Peter Mellinger, Vy Doan, Jeffrey Allen, and Fabien Jammes

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