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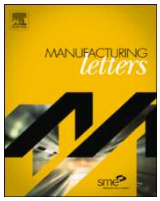

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Direct Writing of Microheater for Studying Plant Thermal Biology

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Abstract

Microheaters have drawn extensive attention for their substantial applications in thermotherapy, gas sensors, thin film preparation, biological research, etc. In plant physiology, uncovering the mechanisms by which plants sense and respond to environmental temperature fluctuations will help us better understand the impact of climate change on crop yield and ecosystem resilience. Currently, microheaters with long-term heating capability have rarely been applied to investigate plant thermal responses. In this study, we applied a direct writing technique to fabricate microheaters suitable for studying plant thermal biology with silver conductive ink. The optimal printing conditions and the heating performance (e.g., stability, durability, reusability) of the printed heaters were thoroughly characterized. The printed microheaters can provide stable and constant heating to plant organs for over four days. When placed near plant leaves to create localized heating, the microheater could successfully activate the expression of a thermoresponsive marker gene in plants. These results demonstrate the potential of applying printed microheaters to study plant thermal biology at the organ and tissue level.

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Keywords: Direct writing; Microheater; Plant thermomorphogenesis

1. Introduction

Printed electronics have held a significant share in the electronics industry during the past few decades due to customizability and high-throughput advantages. Many electronic devices, such as thin-film transistors (TFTs) [1], supercapacitors [2], Light-emitting diodes (LEDs) [3], and flexible heaters [4–6], have been explored. Various printing techniques, including screen printing [5], spray coating [7], EHD printing [8], and transfer printing [9], have been applied to fabricate electronics. However, those techniques either require masks or are unsuitable for scalable fabrication. Among all the printing techniques, direct writing is one of the most developed approaches that offer a promising strategy for creating high-complexity parts with high efficiency and low cost. Moreover, DW is simple and generates less waste. The printing ink is extruded through the nozzle during the direct writing process and deposited on the substrate. The ink flow rate is directly controlled by the pneumatic pressure system, and the printing resolution is controlled by the ink flow rate and printing speed. Thus, the direct writing is capable of printing small devices, for example, microheaters.

Microheaters have attracted a lot of attention because of their substantial applications in gas sensors [10,11], microexplosive boiling [12], healthcare [13,14], biological measurement [15], etc. For instance, heat treatment in healthcare is an effective approach to increase blood flow, soften tissues, reduce short-term pain, and accelerate the diffusion of drug molecules that can help control drug delivery [15]. In the sensing application, microheaters can provide a lower heat mass, ease of integration, and good compatibility with other micro-sensors or devices [16]. Among all types of heaters, thin film heaters that can be

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easily embedded in the object or attached to the skin or layer are widely used in various applications, such as local thermotherapy [17], liquid crystal display in cold weather [18], and window defroster [19]. Printed thin-film microheaters with long-term heating capability can benefit different applications. For example, patient-focused long-term mild heat therapy provides a positive mitochondrial adaptation in skeletal muscle [20]. In the case of gas sensors, printed microheater with long-term capability has less thermal and mechanical stress, which increases performance and reliability [21]. Although printed thin-film microheaters with different materials have been manufactured and tested [17,22–24], most research has not explored the long-term heating performance, limiting their potential applications.

In plant physiology, uncovering the mechanisms by which plants sense and respond to temperature fluctuations will help us better understand the impact of climate change on crop fitness and yield. During the past decade, many studies have demonstrated that global warming profoundly affects plant morphology, leading to elongated organs (e.g., stem, root, and petiole), early flowering, and reduced stomatal index, collectively termed thermomorphogenesis [25,26]. Potential plant thermosensors detect moderately increased temperatures and confer thermal responses by triggering chromatin remodeling and massive transcriptome reprogramming [27–32]. Most thermosensory pathways were revealed at the whole-plant level. Yet, their spatiotemporal contributions to thermomorphogenesis were described ambiguously, inadvertently creating a one-size-fits-all illusion of proposed thermal sensing mechanisms. Recent discoveries of organ- and cell-specific thermosensory signaling strongly advocate the existence of both organ/cell-autonomous and -nonautonomous thermomorphogenetic pathways [33–37]. However, environmental temperatures always influence multiple organs and cell layers simultaneously, so it is difficult to investigate the interorgan and intercellular thermal signaling pathways. As a result, despite the identification of several key signaling modules in thermosensory growth and development, the mechanism of interorgan dialogue and intercellular communication that instruct thermomorphogenesis remains poorly understood.

The main challenge has been the lack of a minimally invasive method that can allow us to accurately induce and modulate thermal changes in a specific organ, tissue, or cell. Almost all organ-specific thermomorphogenetic mechanisms were revealed using detached organs, and few tissue-type- or cell- type-specific mechanisms have been reported. Despite the development of microheaters and thermal probes [38–41], most devices cannot be placed onto plant organs due to their overall size or compatibility. Therefore, there is an urgent need for a new methodology for fabricating miniature heating devices and implanting them onto individual organs.

In this work, we have fabricated thin-film microheaters by applying the direct writing technique for the silver conductive ink. The characterizations were performed to optimize the printing parameters and evaluate the performance of printed microheaters. We successfully printed microheaters that can provide stable and constant heating to plant organs for over four days. The microheater-assisted leaf thermal treatment successfully induced the expression of *HSP70*, an early thermoresponsive marker gene in plants. Therefore, our results imply the feasibility of implementing printed microheaters in studying plant thermal biology.

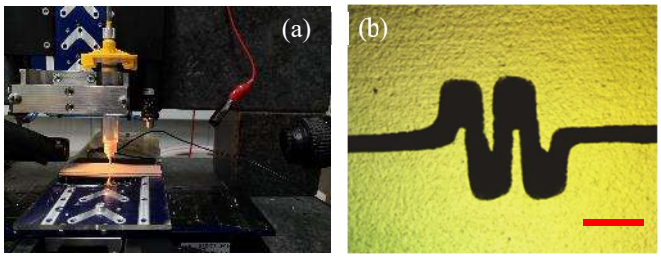

Fig. 1. (a) The direct writing system. (b) Optical microscope image of printed micro-heater. Scale bar: 500 μm

2. Experiment and Method

The printing process was first characterized to obtain the optimal printing parameters (velocity and pressure) for selected ink. Next, microheaters were printed, and the heating performance (heating rate, peak temperature, durability, reusability, and flexibility) was tested. Heating systems with close-loop feedback were developed and characterized before being applied to the plant leaves. Last, the plants were placed in the chamber to study the temperature reaction.

2.1. Direct printing system and printing material

The direct writing system includes a high-precision three-axis motion system and a pneumatic dispensing system. Fig. 1a shows the setup of the direct writing. The pneumatic dispensing system is connected to the syringe to provide back pressure for the ink flow. The precision motion system is placed on an optical table to reduce environmental vibration. It controls the printing nozzle and stage movement in all three axes with an accuracy and repeatability of 50 μm. A camera with a resolution of 1.6 μm is used to monitor the printing process, and the printed features were characterized using the optical microscope and multi-meter. To prevent the nozzle from clogging, the printing material was prepared by mixing silver ink with the Poly(ethylene oxide) (PEO) solution at a volume ratio of 1:1. The silver ink material (Mac Silver) has a solid loading of 60%. The PEO powder with an M_v of 1,000,000 (Sigma-Aldrich) was first diluted with Deionized (DI) water to form the 4% (w/v) PEO solution and then mixed with silver ink. The nozzle has an inner diameter of 160 μm and an outer diameter of 250 μm. A standoff distance of 100 μm was selected for the printing process.

2.2. Fabrication and characterization of microheaters

We first characterized the printing process to determine the best parameters for printing microheaters. Two printing parameters were adopted to control the process: applied pressure and printing speed. The pressure was expected to control the ink flow rate, and the printing speed was used to control the line width of the printed feature. The printed features were observed under the optical microscope. The optimal parameters were selected for the printing of

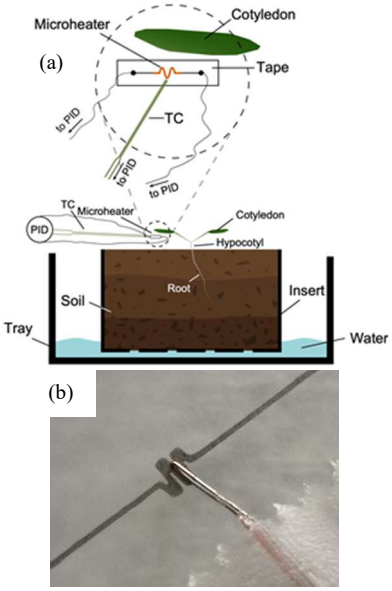

Fig. 2. (a)The schematic of built heating system for plant. (b) Optical image of thermocouple and heater.

microheaters. Small-size heaters can create localized heating for plants, which is essential for studying plant thermal responses in a specific area. Considering that most of the leaves used in this study have a dimension of 2-3 mm x 1 mm, the microheaters with a size of 1 mm x 1 mm were printed. A flexible substrate was needed to attach the heater to the plant leaf conformally. Regular magic tape (3M) was used as the flexible substrate for printing. The tape was first placed on the glass substrate and removed after the printing. Fig. 1b shows the optical image of a printed thin film microheater. The snake pattern was selected as the main component of the heater, and two connecting lines were also printed to connect the heater to external power and control devices.

The experiments were performed to characterize direct writing and obtain the best parameters for a stable process. The printed microheater was characterized in the Percival chamber before being placed under the leaf. Critical factors (stability, durability, and flexibility) were used to evaluate the heating performance of the microheater. The printed heater was first washed under purified water to remove PEO. To characterize the performance of the printed microheater, copper wires, a proportional–integral–derivative (PID) controller (OMEGA CNPT-220), and a thermocouple (OMEGA type E with a 0.08mm wire diameter) were connected to the microheater. The temperature output in PID was set to the desired value, and the thermocouple was placed on top of the heater to provide real-time temperature measurements. The PID was connected to a computer and controlled by software. The software can record data on power consumption and actual temperature from the thermocouple with a customized refresh rate. To examine the stability and durability, the heater was set to 27°C for four days and 42°C for one day, and the actual temperatures on the heaters were recorded. To test the flexibility and reusability of the heater, we conducted a bending fatigue test. The bending radius was set to 2 mm, and a motion stage was used to perform the bending process.

2.3. Heating system for studying the plant thermal responses

Arabidopsis thaliana (Arabidopsis) ecotype Col-0 and *Brassica rapa* ecotype RCBr (Wisconsin Fast Plants Standard) was used in the microheater-assisted thermal studies. Seeds were surface sterilized with diluted bleach (3% sodium hypochlorite) and plated on half-strength Murashige and Skoog media with Gamborg's vitamins, 0.5 mM MES (pH 5.7), and 0.8% (w/v) agar (Caisson Laboratories). Seeds were stratified in the dark at 4°C for 3-5 days before growing in an LED chamber (Percival Scientific), of which the temperature was set to 20°C. Four-day- and five-day-old seedlings were used for thermal treatment. The microheaters were directly placed either near the tip or at the abaxial surface of the leaves. The heater and other connected devices were placed in the Percival chamber, similar to the characterization process. A reference set of plants that were connected with inactive heaters was prepared and placed in the same chamber. Two plant sets have similar heights and ages. The PID temperature was set to 27°C for Arabidopsis or 37°C for *B. rapa*, and the heating time was set to four days. The real temperature data was recorded by PID controlling software. The overall heating system and the heater with thermocouple are shown in Fig. 2.

2.4. RNA extraction and quantitative reverse transcription-PCR

To examine the thermal response triggered by the printed microheater at the molecular level, total RNAs were extracted from Arabidopsis and *B. rapa* seedlings. Two sets of treatments were performed: 1) in the first set, 4-day-old Arabidopsis seedlings were treated with active or inactive (mock) microheaters at the zeitgeber time 8 (ZT8; eight hours after the light was turned on), and samples were harvested at ZT12; 2) in the second set, 5-day-old *B. rapa* seedlings were treated with active or inactive microheaters at ZT4 and harvested at ZT6. Only one of the two cotyledons (embryonic leaves) of each seedling was treated with the active or inactive microheater. Three samples were collected from each treated seedling—the cotyledon treated with the microheater (either active or inactive), the untreated cotyledon, and the remaining tissue that includes the hypocotyl (embryonic stem) and root. Total RNAs were extracted using the Quick-RNA MiniPrep kit with on-column DNase I digestion (Zymo Research). cDNA synthesis was performed with the Superscript III First Strand cDNA Synthesis Kit (ThermoFisher Scientific). For quantitative reverse transcription-PCR (RT-qPCR), diluted cDNA was mixed with FastStart Universal SYBR Green Master (MilliporeSigma) and gene-specific primers. RT–qPCR reactions were performed in triplicate with a Qiagen Rotor-Gene Q 5Plex real-time cycler (Qiagen, Germantown). Transcript levels of each gene were calculated relative to that of *PP2A* (Arabidopsis) or *Brara.K01792* (*B. rapa*) by the $2^{-\Delta\Delta Ct}$ method. Bar charts were generated using Prism 9 (GraphPad Software). Primers were: *PP2A* (AT1G13320), TATCGGATGACGATTCTTCGTGCAG and GCTTGGTCGACTATCGGAATGAGAG; *Brara.K01792*

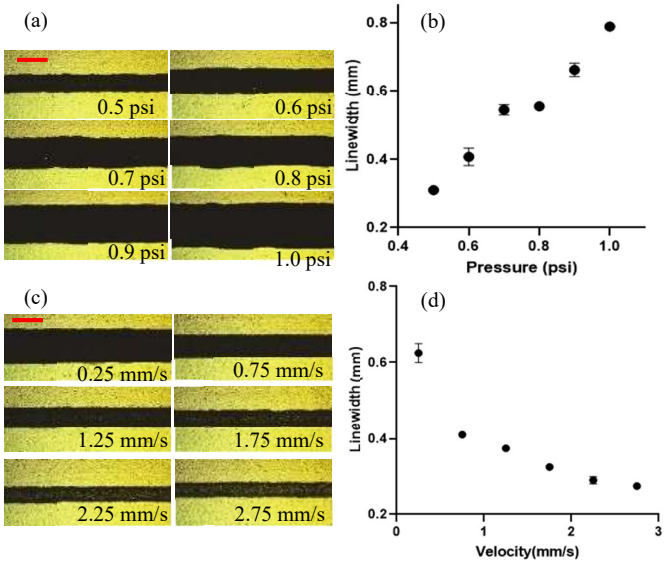

Fig. 3. (a) The optical microscope image of printed silver conductive lines with different pressure. (b) Relationship between the printed linewidth and applied pressure. (c) The optical microscope image of printed silver conductive lines with different printing speed. (d) Relationship between the printed linewidth and applied printing speed. Scale bar: 500μm

(BraA03g049110.3C), TCGTCTTCTCAAATCTCCCTC and GTAACCTTGCCACAGATTCG; *At HSP70-4* (AT3G12580), GAAGTACAAGGCTGAGGATGAAGAAC and CTTCTCGTCCTTGATCGTGTTCC; *B.rapa HSP70-1* (BraA01g038810.3C), ATCATCGCCAACGATCAAGG and AGACGGTGTTGGTAGGGTTC. The plant studies have been performed 6 times in Arabidopsis and 3 times in *B. rapa,* and similar results were obtained. Representative results from 8 (Arabidopsis) and 2 (*B. rapa*) biological replicates were shown.

3. Results and discussion

As we know, increasing the back pressure will accelerate the ink flow rate. A higher flow rate will result in a larger filament diameter when keeping the same printing speed. Fig. 3a and b show the result of the printed silver filaments under different pressures. All the filaments exhibited smooth edges. When applying a small amount of pressure (0.5 psi), thinner filaments with a diameter of 0.31 mm can be obtained. After increasing the pressure to 1 psi, filaments with a diameter of 0.78 mm were observed. Printing speed is the other critical factor affecting the printing process and filament quality. With the same ink flow rate, a higher speed will create narrower filaments, while a slower speed will bring wider filaments (Fig. 3c and d). Moreover, the slow printing speed will result in material collections around the nozzle, which brings challenges to controlling the linewidth. From the test results, higher pressure will get a wider line, while faster velocity will bring a thinner line. To get the thinnest line, higher velocity, and lower pressure should be selected. In this experiment, the final selected printing speed and pressure were set to 2.75 mm/s and 0.5 psi to ensure stable printing with good resolution.

To test the heating performance, we focused on stability and durability in this study. Plants are susceptible to environmental temperatures. Even a slight temperature difference will dramatically change plant growth and development. Therefore, the temperature fluctuation of the microheater should be

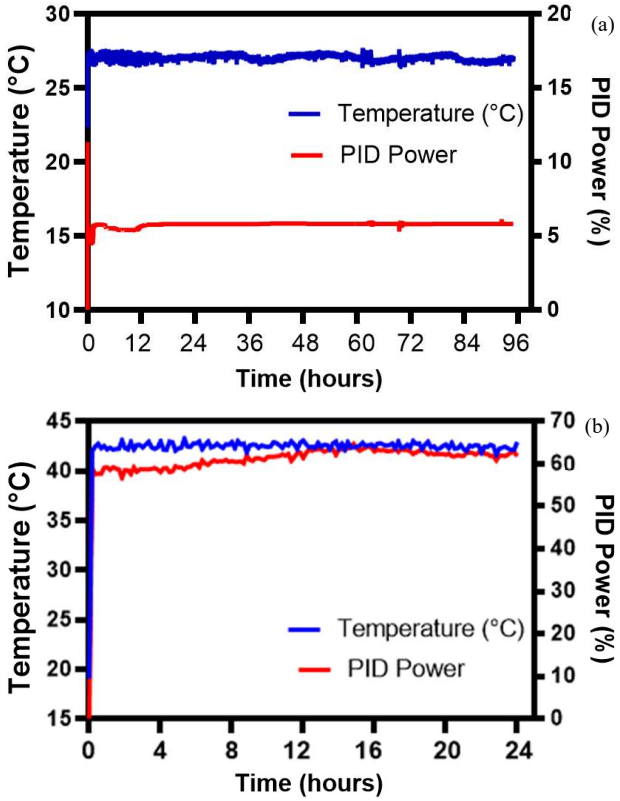

Fig. 4. (a) The heater with a 27°C heating for 4 days. (b) The heater with a 42°C heating for 24 hours

minimized to provide stable heating for the leaf. The long-term heating capability of the heater is another critical factor. The heater should provide stable long-term heating during the thermal treatment for the plant, which will allow the plant to generate accurate and sufficient signals that are necessary for significant changes in the plant morphology. Fig. 4a shows the output heating temperature and power consumption during the four-day treatment period when the PID was set to 27°C. During the testing period, the peak-peak temperature was within 1°C, and the power consumption remained at a constant level, demonstrating good heating stability. Investigating how plants respond to heat stress is also an important topic for agricultural development and ecosystem resilience because extremely high temperatures, such as heat waves, have imposed great impacts on crop yield and biodiversity [42,43]. Therefore, we also tested the long-term stability of the printed heater at higher temperatures. Fig. 4b shows the 24-hour heating performance of the heater when the target temperature was set to 42°C. The result shows an excellent and stable heating performance even at this higher temperature, which illustrates that the printed heater can provide long-term, stable heating for the plant at a wide temperature range.

Fig. 5a shows the result of resistance changes in the microheater under different bending cycles. Even with 1000 times bending, only a slight difference (< 4Ω) in the resistance was observed, indicating the great potential of reusing these printed microheaters. Fig. 5b, c, and d show the heating rate, maximum heating temperature, and the repeated heating performance of the heater, respectively. The heater temperature rose from 20°C to 42°C in 150 seconds, showing an excellent heating rate that can overcome any disturbances and maintain

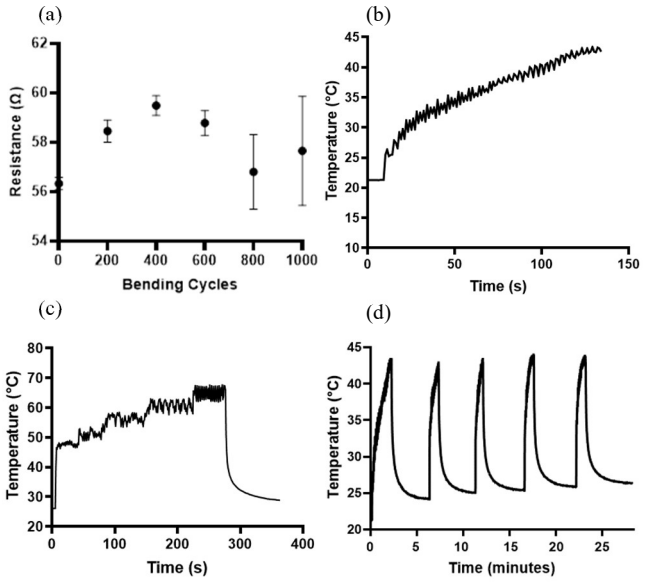

Fig. 5. Results of the (a) resistance of micro-heater with different bending cycles. (b) Heating rate. (c) Maximum heating temperature, and (d) Repeat heating cycle.

a stable temperature. The maximum temperature of the printed heater was above 60°C, which was enough to provide the extreme temperature condition for studying the plant thermal response. During on-off operations within 25 mins, for each repeated heating cycle, the duration of the heating and cooling process of the heater was similar, which showed superior repeatability of the printed heater.

To further test the heater performance on plants, we treated Arabidopsis and *B. rapa* seedlings with these microheaters and examined the expression levels of *HEAT SHOCK PROTEIN 70* (*HSP70*), an early high-temperature-induced gene. It has been well demonstrated that the expression of Arabidopsis *HSP70-4*

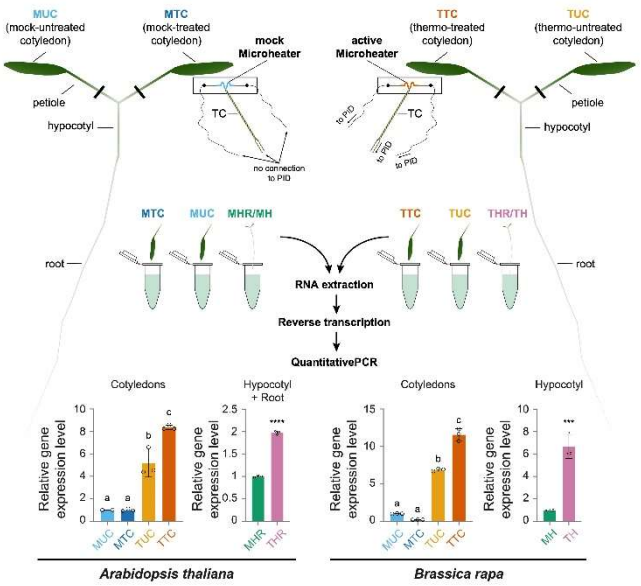

Fig. 6. Microheater triggers the expression of thermo-responsive genes in plant leaves. Young Arabidopsis and *Brassica rapa* seedlings grown at a constant 20°C were treated with either a PID-controlled active microheater (right) or a mock microheater without PID connections (left). Samples were harvested for RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to examine the expression levels of the thermo-responsive genes *At HSP70-4* and *B. rapa HSP70-1*. See the detailed experimental design in the main text.

could be induced by warm temperatures (e.g., 27°C) [44], and that of *B. rapa HSP70-1* could be enhanced by high temperatures (e.g., 37°C) [45].

To accurately evaluate the effect of microheaters on plant thermal response, we performed a mock treatment in which a group of plants was treated with inactive microheaters (not connected with PID; Fig. 6). For both the experimental and control groups, we placed the heater near only one of the two cotyledons in each plant and harvested the treated and untreated cotyledons separately after heating Arabidopsis at 27°C for 4 hours and *B. rapa* at 37°C for 2 hours. The remaining organs after dissection, which included hypocotyl and root (for Arabidopsis) or hypocotyl only (for *B. rapa*), were also collected. Therefore, we obtained six samples, four of which were cotyledons and two were hypocotyls and roots or hypocotyls only, from each batch of assays (Fig. 6). After RNA extraction and reverse transcription, we compared the *HSP70* expression levels among the four cotyledon samples and between the two hypocotyl & root or hypocotyl only samples using quantitative PCR assays. When treating Arabidopsis at 27°C for 4 hours and *B. rapa* at 37°C for 2 hours, we observed a highly induced expression of *HSP70* in the thermo-treated cotyledons (TTC) when compared with mock-treated and -untreated cotyledons (MTC and MUC) from the control plants (Fig. 6). This result is consistent with previous studies (Zhu et al. 2016; Tabusam et al. 2022), demonstrating the effectiveness of the printed microheaters on triggering plant thermal responses.

Surprisingly, we also observed that the *HSP70* transcript level was induced in the thermo-untreated cotyledon (TUC) when compared with MTC and MUC, although the induction was significantly lower than that in TTC (Fig. 6). Moreover, we could also observe the inductin of *HSP70* expression in the thermo-treated hypocotyl and root (THR) or hypocotyl only (TH) when compared with the mock-treated ones (MHR or MH) (Fig. 6). These data imply that a mobile signal will be generated when the cotyledon senses temperature changes and the signal can be transduced to distal organs (e.g., the other cotyledon and hypocotyl) to activate the expression of thermal response genes. We will explore the molecular mechanisms leading to this interesting phenomenon in future studies.

4. Conclusion

In this study, we applied the direct writing technique to fabricate microheaters for studying plant thermal response with silver conductive ink. The characterization processes were conducted to find the optimal printing condition to fabricate the microheater. The heating performance, including stability, durability, reusability, heating rate, cycle heating, and duration, was characterized. The printed heater can obtain a stable heating performance for four days at a temperature of 27°C. The microheaters also induced thermal stress responses at the transcriptional level. The plant thermal treatment results show the potential for studying plant thermal biology with these printed microheaters. The future work includes testing heaters

with different designs and sizes, and studying the thermal responses of other plant organs, such as root and stem.

Acknowledgments

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