Chapter 20

Investigating Circadian Gating of Temperature Responsive Genes

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Abstract

Understanding gene expression dynamics in the context of the time of day and temperature response is an important part of understanding plant thermotolerance in a changing climate. Performing "gating" experiments under constant conditions and light-dark cycles allows users to identify and dissect the contribution of the time of day and circadian clock to the dynamic nature of stress-responsive genes. Here, we describe the design of specific laboratory experiments in plants (*Arabidopsis thaliana* and bread wheat, *Triticum aestivum*) to investigate temporal responses to heat (1 h at 37 °C) or cold (3 h at 4 °C), and we include known marker genes that have circadian-gated responses to temperature changes.

Key words Phase, Gating, Entrainment, Temperature stress, Transcript abundance

1 Introduction

A fundamental property of the circadian clock is to control the expression of a repertoire of genes involved in key biological processes [1, 2]. Modulation of the dynamics of gene expression responses to fluctuating daily environmental stimuli or stressors, according to the time of day, is referred to as circadian or time of day gating of the response [1]. Depending on the context of the organism and stimuli, the concept of circadian gating can differ. For example, there is circadian gating of the emergence of adult *Drosophila* to specific times of the day, and circadian control of cell division can be confined to specific periods in a day [3, 4]. Circadian gating also acts on entrainment pathways. For example, the clock can gate environmental inputs such as light and temperature to appropriately synchronize and calibrate the phase of the oscillator [5–8]. In another context and increasingly demonstrated in plants, circadian gating or time of day control often considers the application of a short treatment (1–3 h) at different times of the day, and interpreting the subsequent gene expression responses based on

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changes in transcript abundance or other readouts such as luciferase reporters of promoter activity and fluorescent proteins [9–21]. Both transcripts that are cycling (rhythmic) and non-cycling (non-rhythmic) in the absence of the stimulus can have a gated response to a given stimulus, regardless of whether the plants are cultivated under 24 h cycles of light and dark (diel cycles) or under conditions of constant light and temperature (free running conditions) (Fig. 1). Whilst the gating of stimulus-induced responses under cycles of light and dark might be regulated by the circadian clock, experimentation under constant light conditions or with clock mutants becomes necessary to confidently assign the gating response to the circadian clock. Understanding the concept of gating as it relates to stress responses is critical (i) in the context of climate change, because temperature variations can have negative impacts on plant growth and yield depending on the time of day; (ii) in unraveling the dynamics of gene regulatory networks involved in stress response and tolerance; (iii) in helping to interpret datasets generated by different research groups for which stress was applied at different times of the day. In this methods chapter, we provide a framework for designing an experiment to investigate circadian gating of temperature responses in plants, using *Arabidopsis thaliana* (*Arabidopsis*) and *Triticum aestivum* (hexaploid bread wheat) as examples in relation to heat (37 °C) and cold stress (4 °C), respectively [18, 19]. In addition, we provide examples of genes that can aid with the validation of a successful gating experiment.

2 Materials

All Petri dishes and reagents are prepared using sterile deionized water and under sterile conditions where appropriate. Calculate carefully the number of plants that are needed for the experiment. We recommend harvesting at least one spare sample per time point and condition, in case of sample loss during extraction. With large experiments involving long time courses, there is always a risk of sampling the wrong material, especially where several genotypes are used. One solution can be to label the plates or plant pots with colored tape, using different colors per genotype, because this can be more instantly recognizable than text on labels.

2.1 Arabidopsis Plant Growth

1. 1 × MS medium plates: 4.3 g/L Murashige and Skoog Basal Salts (Caisson Labs MSP01-50LT), 1.5% (w/v) Sucrose (Fisher Scientific S5-500), pH to 5.7–5.9 using KOH, add 8 g/L agar (Sigma-Aldrich A1296). Autoclave and prepare circular petri dishes (100 × 15mm, VWR 25384-088) containing 25–30 mL of MS media supplemented with 1.5% (w/v) sucrose (*see* **Note 1**).

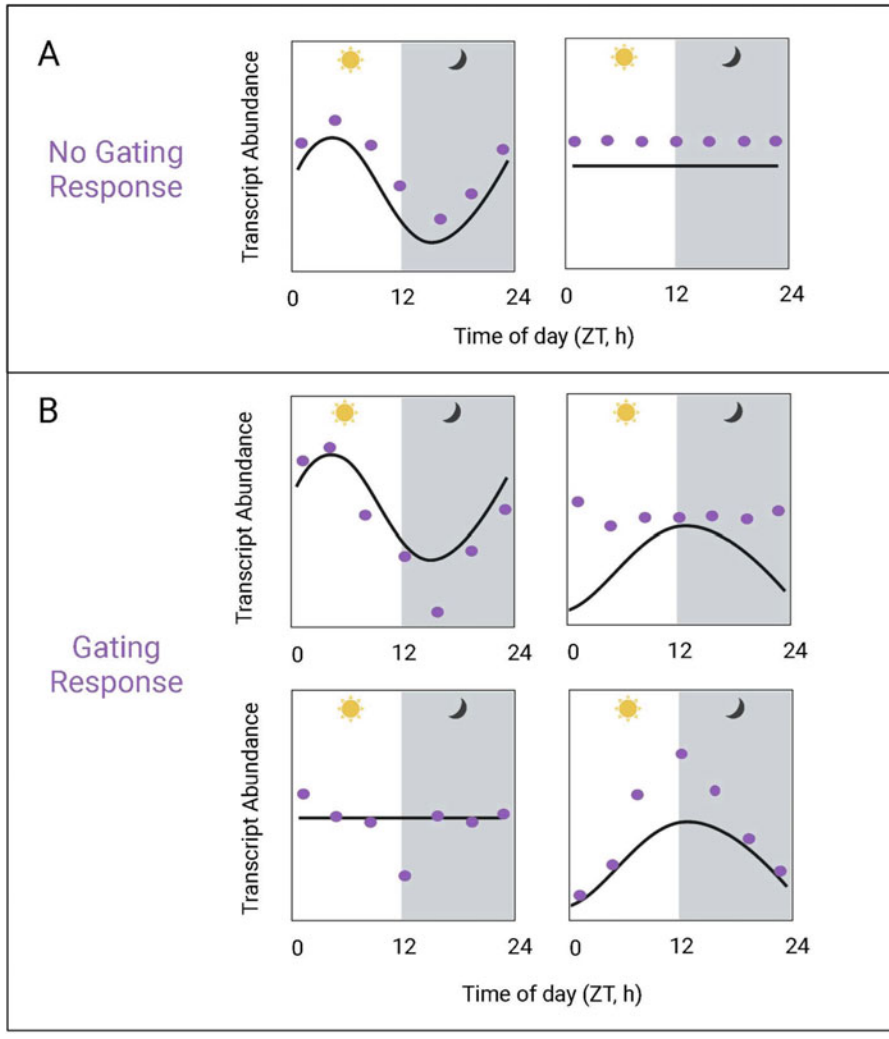

Fig. 1 Schematic representations of circadian gating responses. (**a**) Transcripts that either have a circadian rhythm (left panel) or do not have a circadian rhythm (right panel), and do not undergo circadian gating of their response to a stimulus. (**b**) Examples of circadian gating of a response to a stimulus. In some examples, there is a circadian rhythm in the magnitude of the response to a stimulus, where the response also oscillates with a 24 h rhythm. Other examples do not have a cycling pattern of response. Black lines represent the transcript abundance under control conditions. Purple circles indicate the relative transcript abundance after a temperature stimulus, given separately at each time point. The white area represents the subjective day (ZT0-12, yellow sun), and the gray areas (ZT12-24, moon symbol) represent the subjective night, under conditions of constant light and constant temperature. Time of day is represented as Zeitgeber or hours (ZT, h). Image created with Biorender

2. Grade 1 circular qualitative filter paper standard grade, sterilized by autoclaving (90 mm, VWR 28450-081). To autoclave, wrap in aluminum foil and place in an autoclavable vessel. Working in a laminar flow hood, use sterile forceps to place a single filter paper on the MS plates (*see* **Note 2**).

3. Plant Growth Chambers for plates.
4. Bleach (Sodium hypochlorite).
5. Hydrochloric Acid (HCl).

2.2 Wheat Plant Growth

1. Filter paper (clean, not sterilized).
2. 24-cell disposable trays for horticultural use
3. Wheat growing medium: 65% (v/v) peat, 25% (v/v) loam, 10% (v/v) horticultural grit, 3 kg m^{-3} dolomitic limestone, 1.3 kg m^{-3} Haifa Multimix fertilizer 14:16:18 (N:P:K) (Haifa Group), 3 kg m^{-3} Osmocote Exact 15:9:11 3/4 (N:P:K) slow-release fertilizer (ICL Agriculture). Compost can be mixed and trays filled by hand, although at the John Innes Centre we use automated growing medium preparation and tray filling equipment for consistency.
4. Growth chambers with sufficient space for cultivation of bread wheat seedlings.

2.3 Plant Tissue Collection, RNA Isolation, Complementary DNA (cDNA) Synthesis, Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qPCR)

1. Sterile 2.0 mL microcentrifuge tubes (Seal-Rite® 2.0 mL Microcentrifuge Tubes USA Scientific #1620-2700).
2. Liquid Nitrogen.
3. Stainless steel/metal beads (BioSpec Products 11079132ss).
4. Tissue Lyser (Retsch Mixer Mill MM400 UX-04182-09).
5. Plant RNA isolation kit for *Arabidopsis*: GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific K0801).
6. Plant RNA isolation kit for wheat: Machery-Nagel Nucleospin RNA Mini Kit (Thermo Fisher Scientific 740955.50). Alternative kits might be suitable, depending on the laboratory preference.
7. DNase I, RNase-free (1 U/μL) (e.g., Thermo Fisher Scientific EN0521, or as supplied with Machery-Nagel kit).
8. cDNA synthesis kit for *Arabidopsis*: iScript™ cDNA Synthesis Kit, which includes oligo(dT) and random primers as part of the 5X iScript Reaction Mix (Biorad 1708890),
9. cDNA synthesis kit for wheat: High-Capacity cDNA Reverse Transcription Kit, with random primers (Thermo Fisher Scientific, 4368814),
10. qPCR reaction mix for *Arabidopsis*: Maxima SYBR Green mix (Thermo Fisher Scientific K0252),
11. qPCR reaction mix for wheat: qPCRBIO SyGreen LO-ROX RT-qPCR reagent (PCR Biosystems, selecting reagent according to qPCR machine model),
12. RNaseZap™ RNase Decontamination Solution (Thermo Fisher Scientific AM9780).

13. Filter Pipette Tips (e.g., USA Scientific TipOne RPT ultra-low retention filter tips, 1000uL #1182–1830; 200ul #1180–8810; 10ul #1181–3810).
14. Real-Time PCR Detection System (e.g. CFX96 or CFX384 Real-Time PCR Machine Biorad).
15. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Biorad HSP9601) or TempPlate polypropylene 384-well PCR plate (USA Scientific #1438–4790).
16. Microseal "B" PCR Plate Sealing Film, adhesive, optical (Bio-rad MSB1001).
17. Arabidopsis qPCR housekeeping gene primers: *IPP2* (IPP2-F: 5′- CTCCCTTGGGACGTATGCTG-3′; IPP2-R: 5′- TTGA ACCTTCACGTCTCGCA-3′) and *PP2A* (PP2A-F: 5′-TAAC GTGGCCAAAATGATGC -3′; PP2A-F: 5′- GTTCTCCACAACCGATTGGT-3′).
18. Wheat qPCR housekeeping gene primers: *TaHK4* (TaHK4-F: 5′- TCTAAATGTCCAGGAAGCTGTTA -3′; TaHK4-R: 5′- -CCTGTGGTGCCCAACTATT-3′).

2.4 Candidate Marker Genes with Circadian-Gated Responses

1. First, it is critical to confirm that the appropriate rhythmicity is observed in the control samples following collection and tissue processing, using a couple of known clock genes and qPCR analysis as described below (*see* **Note 3**). To validate the successful implementation of a gating experiment, several marker genes can be used to confirm the dynamics of time of day and temperature-dependent changes in transcript accumulation. As an example, *Arabidopsis GIGANTEA (GI)* transcript abundance has a circadian gated response to heat stress (1 h at 37 °C; Fig. 2a). GI is clock-regulated and functions in the photoperiod flowering pathway [22, 23]. Furthermore, in the context of temperature and gating, *GI* transcript accumulation increases at high ambient temperatures (28 °C) as compared to the cooler temperatures of 12 °C at dawn and is also cold-responsive [24, 25]. In recent years, several studies have reported a functional role for GI in the gating of light signals, hormone sensitivity, and shade avoidance responses [26–28]. Of note, in fundamental studies, GI has been reported to participate in temperature compensation of the clock, primarily at higher temperatures [23, 29]. Together, this implicates an important role for GI as a marker for abiotic stress responses and environmental changes (*see* **Note 4**). Likewise, there are excellent marker genes for the validation of cold-temperature responses in wheat. One example is the orthologs of the *CBF* gene family in wheat, which are strongly cold-induced and undergo circadian gating of their response to cold [11]. In wheat, we have used *TaCBFIIId_D19* (Fig. 2b).

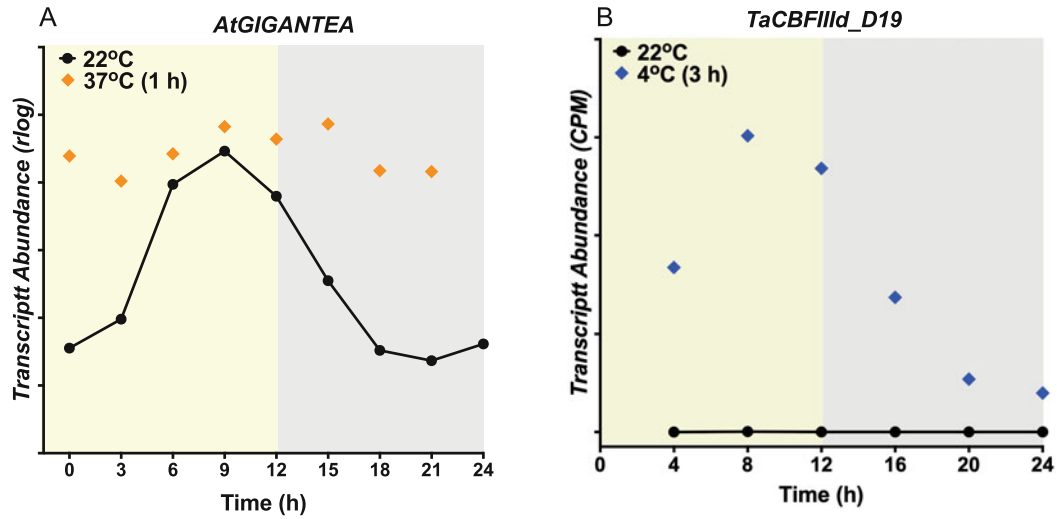

Fig. 2 Examples of genes that have circadian gating of their responses to acute temperature stimuli. (**a**) Arabidopsis *GIGANTEA* transcript abundance (*Rlog normalized counts*). Data are means for N = 3 biological replicates. 37 °C (orange diamond) and 22 °C (black line and circles). Each exposure to heat stress (37 °C) was for 1 h. Time (h) represents (ZT 48–77). *Arabidopsis* data reproduced from Bonnot et al., 2022 [18] with permission from Oxford Academic Press. (**b**) Wheat *CBF (TaCBFIIId_D19)* transcript abundance (*CPM, counts per million*). Data are means for N = 3 biological replicates. 4 °C (blue diamond) and 22 °C (black circles). Each exposure to cold stress (4 °C) was for 3 h and ended at the time shown on the x-axis. Time (h) represents (ZT 28-48). Data extracted from European Nucleotide Archive dataset PRJEB56923 (collected by Graham CA, Paajanen P, Edwards KJ, Dodd AN, within [19]). Yellow/gray shading = subjective day/night

3 Methods

Growth and experimental procedures are indicated where applicable. Otherwise, all procedures can be performed at room temperature.

3.1 Plant Growth and Tissue Collection

For *Arabidopsis*

1. Surface sterilize *Arabidopsis* seeds (100 mL of bleach +3–4 mL of Hydrochloric Acid (HCl)) in a glass desiccator jar for at least 3 h (*see* **Note 5**).
2. In a laminar flow hood, sprinkle the sterilized seeds onto the filter paper containing MS plates attempting to disperse the seeds evenly across the filter paper. Stratify the seeds for 2–4 days at 4 °C in the dark.
3. Transfer plates to a growth chamber (Percival, Perry, IA) with specified conditions depending on the plant. For *Arabidopsis*, grow seedlings in 12 h light (Fluorescent Tube, ~130 μmol m^{-2} s^{-1}): 12 h dark and constant 22 °C for 10 days. If using circadian conditions, on Day 11, transfer plates to conditions of

constant light (Fluorescent Tube, 80–100 μmol m^{-2} s^{-1}) and temperature for 2 days (*see* **Note 6**).

For Wheat

4. Place filter paper inside a petri dish and evenly spread wheat seeds over the filter paper. Moisten filter paper pieces with tap water so the entire paper is damp but seeds are not floating. The filter paper should be clean but does not need to be sterile.
5. Stratify the wheat seeds on filter paper at 4 °C for 3 days, then transfer to 22 °C in darkness for two days to germinate.
6. Pot germinated seedlings into a cereal cultivation mixture (see Materials and Methods for composition), in 24-cell horticultural trays.
7. Cultivate for 12 days under cycles of 12 h light/12 h darkness, at 22 °C, in MLR-352 growth chambers (Panasonic, Osaka, Japan; white light supplied by LED tubes). Rotate trays every three days to ensure even light distribution and consistent growth.

3.2 Temperature Stress and Sample Collection

For *Arabidopsis*

1. For heat shock gating of *Arabidopsis* seedlings, transfer plates to a growth chamber preset to 37 °C for 1 h every 3 or 4 h over a 24 h period. Control (non-stressed) plates should be kept at 22 °C (*see* **Note 7**).
2. Immediately collect whole seedlings in 2 mL centrifuge tubes from heat-stressed plates following the 1 h treatment and from the control plates. Snap freeze seedlings containing centrifuge tubes in liquid nitrogen (N_2). Tissue can either be immediately ground into powder or stored at −80 °C until processed. If using a grinding mill, include 3 metal beads in the tube before adding the sample (*see* **Note 8**).

For Wheat

3. For cold temperature treatment of wheat plants, transfer 14-day old seedlings (at Zadok Stage GS1.2) to conditions of continuous light and temperature (22 °C), 24 h before sampling begins. This ensures that the transitory effects of the final dawn are absent from the data.
4. After 25 h of constant light conditions, transfer three individual plants to 4 °C, with three control plants remaining at 22 °C. Both remain under white light (*see* **Note 9**).
5. After 3 h exposure to 4 °C, sample the tissue. Collect 3 cm of tissue from the second leaf into a pre-labeled Eppendorf tube

loaded with 2 or 3 metal (tungsten) beads. Freeze immediately in liquid N_2, and store at −80 °C for later processing. Repeat this process for the remaining two cold-treated individuals, and also for three control-temperature individuals.

6. Repeat this process after 29 h of constant light conditions and every further 4 h, for at least 24 h of sampling.

3.3 Total RNA Isolation

1. Clean the work area with RNaseZap prior to the RNA isolation procedure (*see* **Note 10**).
2. For *Arabidopsis*, grind seedlings using a tissue lyser (also known as a ball mill or bead beater) at a frequency rate of 28 Hz for 1 min or a mortar and pestle. Both the tissue lyser racks and mortar and pestle should be chilled with liquid nitrogen prior to grinding and kept cold throughout. For wheat, load Eppendorfs containing samples into a tissue lyser rack that has been stored at −80 °C. Grind wheat samples using a tissue lyser for 2 min at 28 Hz.
3. Isolate total RNA from *Arabidopsis* using the GeneJET Plant RNA Purification Mini Kit (*see* **Note 11**), and from wheat using the NucleoSpin RNA Mini Kit (Machery-Nagel, Düren, Germany), applying an on-column DNase treatment.
4. Check total RNA concentration using a nanodrop. A minimum of 150 ng/μL of total RNA is recommended for the next steps.
5. Check RNA integrity by running 1 μL of total RNA on a 1.5% agarose gel. Intact high-quality total RNA will have sharp and distinct 28S and 18S rRNA bands in a ~ 2:1 ratio (28S:18S) (*see* **Note 12**). Alternatively, RNA integrity can be assessed using a 2100 Bioanalyzer System (Agilent, Santa Clara, USA), confirming RIN scores >6.

3.4 Quantitative Real-Time PCR

1. For *Arabidopsis*, treat 1 μg of total RNA with DNase I to remove DNA contamination following the manufacturer's protocol. For Thermo Fisher DNase I, incubate the reaction at 37 °C for 30 min. Add 1 μL of 50 mM EDTA and incubate at 65 °C for 10 min (*see* **Note 13**). Alternatively, the wheat RNA isolation protocol uses an on-column DNA digest.
2. For quantitative real-time PCR, perform complementary DNA (cDNA) synthesis using the preferred kit. For iScript cDNA Synthesis Kit (Bio-Rad), add 4 μL of 5× iScript Reaction Mix, 1 μL of iScript RT, and 4.1 μL of kit provided H_2O to the 10.9 μl of DNase treated total RNA. Incubate the cDNA synthesis reaction in a PCR machine as follows: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and hold at 4 °C.
3. Dilute the cDNA by adding 180 μL of sterile H_2O.

4. Perform quantitative real-time PCR using an SYBR Green kit such as Maxima SYBR Green mix (Thermo Fisher Scientific). Include at least 2 known and well-established housekeeping genes such as *Protein Phosphate 2A (PP2A)* and *isopentyl-diphosphate delta-isomerase II (IPP2)* to be to normalize controls for relative transcript abundance (*see* **Note 14**). For wheat, *TraesCS5A02G015600 (Traes_5AS_019ECA143)* that encodes an ion exchange channel can be used as a stable housekeeping gene (as well as this gene, other candidates are also proposed in Borrill et al., 2016 [30]). Ensure appropriate tests for efficiency of amplification and primer specificity are conducted before embarking on the analysis of large numbers of samples.

3.5 RNA-Sequencing

1. For genome-wide studies and questions concerning circadian gating, mRNA sequencing can be performed on the collected samples using a variety of published approaches [16, 19, 31].

3.6 Data Interpretation

1. A variety of strategies can be considered for the interpretation of the data. First, it is important to confirm that transcripts known to be rhythmic under control temperature conditions have the expected circadian rhythm. This is necessary to ensure that the sampled plant material had been entrained to environmental conditions, before investing time and research funds in further sample analysis. To subsequently test the hypothesis that a transcript undergoes circadian gating of its response to a stimulus, it is necessary to test whether there is a 24 h fluctuation in its response to the stimulus (Fig. 1). At a straightforward level, it is possible to test whether the response to the stimulus has a circadian rhythm in its magnitude. This approach is informative when the number of time points tested is relatively small. For example, if the transcript abundance of a selected gene increases or decreases in magnitude at one or more but not all time points, it could be considered to be gated. This analysis can be elaborated, for example, to test whether the magnitude of response to the stimulus itself has a circadian rhythm. One such approach is to calculate the difference, for each time point, between the transcript abundance at the control temperature and after the temperature treatment. It is then possible to test whether this time series of differences has a circadian rhythm, using a tool such as MetaCycle or BioDare2 [32–34]. Downstream experiments can then be designed based on the gating of the temperature response of the gene(s) of interest, focusing on regulatory players or functional relevance. The approach also enables researchers to subsequently select appropriate times of day to perform a temperature treatment to obtain the most appropriate and reproducible response.

4 Notes

1. The inclusion of sucrose in cultivation media is optional and somewhat laboratory-dependent, but an important consideration and decision because exogenous sucrose can affect the functioning of the *Arabidopsis* circadian clock [35].
2. The use of filter paper allows easier collection of seedlings and prevents root damage during harvest. Users can choose to grow seedlings on a membrane rather than filter paper, and some users prefer to allow roots to grow into the agar and harvest the aerial parts of the seedlings.
3. Following the collection of the time course of samples and tissue processing, it is strongly recommended to examine by qPCR a morning-expressed and evening-expressed clock gene, to confirm that the samples were collected at the correct times of day, that known clock genes are peaking at the correct times in the control samples, and to confirm that abnormal samples are not present, before investing time in further analysis.
4. Although *GIGANTEA (GI)* is used as a marker gene for gating here, other genes may be more suitable depending on the plant species, developmental age, and nature of the temperature or stress treatment.
5. Other methods of seed sterilization can be used such as 70% (v/v) Ethanol/0.1% (v/v) Triton X-100 solutions. If using Bleach/HCl, it is important that tubes are labeled with a pencil rather than a pen, to prevent bleaching of the labels.
6. Different light and temperature regimes, growth duration, and control conditions can be used, depending on the plant species.
7. The temperature and duration used for heat stress may vary depending on the plant species. Alternative methods to apply heat stress can be used.
8. Other methods to grind tissue finely may be used. It is important that the tissue remains frozen until the start of the total RNA isolation procedure.
9. It is highly recommended that you include additional plants as replicates in the event that plants/samples are compromised during subsequent steps.
10. Other RNA decontamination methods can be used.
11. Other plant RNA isolation kits or Trizol methods can be used.
12. Checking RNA integrity is optional but recommended prior to cDNA synthesis.
13. cDNA synthesis kits from other manufacturers can be used.

14. SYBR green kits from other manufacturers may be used. Both *IPP2* (IPP2-F: 5′- CTCCCTTGGGACGTATGCTG-3′; IPP2-R: 5′- TTGAACCTTCACGTCTCGCA-3′) and *PP2A* (PP2A-F: 5′-TAACGTGGCCAAAATGATGC-3′; PP2A-F: 5′-GTTCTCCACAACCGATTGGT-3′) are commonly used in circadian studies in Arabidopsis. In wheat, *TaHK4* (TaHK4-F: 5′-TCTAAATGTCCAGGAAGCTGTTA-3′; TaHK4-R: 5′-CCTGTGGTGCCCAACTATT-3′) have been used. It is important to validate that your housekeeping gene does not display robust circadian cycling or has altered expression based on the treatment.

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