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Assays to study plant response to high temperature in Arabidopsis: evidence in glutathione biosynthesis mutants.

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Link between our chapter to the book's theme on plant stress memory:

This chapter describes procedures to study plant stress tolerance to different high temperature regimes, including acquired thermotolerance inducing stress memory responses. Evidence of the role of glutathione in these responses is shown.

Abstract

In the context of climate change, the global rise of temperature and intense heat waves affect plant development and productivity. In order to decipher the molecular and physiological mechanism established by plants to adapt to increased temperatures, we and others, have designed different high temperature regimes to mimic as much as possible temperature variations occurring *in natura*. This chapter outlines these thermotolerance assays employed to assess response to high temperature in *Arabidopsis thaliana*. We provide detailed guidelines, including plant age considerations and timing of heat application. Moreover, we introduce new findings showing that the addition of sucrose to the growth medium can artificially enhance thermotolerance, potentially masking stress-related phenotypes. These assays, which measure both basal and acquired thermotolerance, offer a framework for assessing plant heat stress responses in a reproducible and efficient manner. To illustrate some plant response to these different regimes, we compare the response of mutants affected in the biosynthesis of the redox buffer glutathione with wild-type plants.

1. Introduction

Thermotolerance assays are widely used to assess whether a given gene plays a role in heat stress tolerance. Yeh et al. (2012) [1] provided a classification system that has improved communication and reproducibility across studies in plant species. These assays have helped identifying key genes involved in redox signaling during heat stress, as recently reviewed by Hendrix et al. (2023) [2].

Plant adaptation to high ambient temperature as 2-5°C above the optimal temperature range have been described as a process generically called thermomorphogenesis (TM) (Figure 1A) [3]. Physiologically, TM leads to developmental modifications like elongation of the hypocotyl, early flowering or modifications of stomata development. Molecular mechanisms underlying these developmental modifications include gene expression reprogramming, chromatin remodelling or hormone accumulation [3]. For example, the network of transcriptional and posttranscriptional regulators involving the photoreceptor phytochrome B (PHYB) and the transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) play a central role in inducing developmental promoting factors like auxins and brassinosteroids metabolism, flowering, or stomata development genes [4, 5, 6]. TM experiments are generally performed on 5 days-old plantlets grown at 20°C and further subjected to 27°C. After 7 days, measurement of hypocotyl length is used as a proxy to estimate the TM response.

Harsher high temperature regimes can be classified in three different types: basal thermotolerance (BT) (Figure 1B), short- and long-term acquired thermotolerance (SAT/LAT) (Figure 1C), and thermotolerance to moderately high temperature (TMHT) (Figure 1D). The BT test is based on an abrupt increase of the ambient temperature (20–22 °C) to 40–45 °C during a short time period of 0.5–1 h. The SAT and LAT tests include pre-incubations at moderate high temperature (about 1 h at 37°C) called priming followed by a harsher high temperature regime (generally 1–2 h at 44 °C). The interval of time between the priming step and the more severe heat treatment ranges from 2 h for SAT to 2 days for LAT experiments. These latter regimes are designed to study pathways involved in

thermomemory [7]. The priming step is designed to induce the expression of heat stress response genes like *HSP101*, helping the plant to survive to the following severe heat stress. Therefore, mutants like *hsp101* are usually used as control lines in these experiments. Because BT, SAT, and LAT assays involve short treatments, they are generally performed using a pre-heated water bath for a rapid increase in temperature. Finally, the TMHT tests consist of a 33–38 °C treatment for several days (5–8 d). Key actors involved in the response to TMHT are Heat Shock Factors (HSF), which mutants (e.g. *hsfa1qk*) can be used as control lines. BT, SAT, LAT and TMHT treatment are followed by recovery phase at 20°C during 7-21 days, after which period, the viability of plants is estimated, as described in the following sections.

In order to illustrate these methods, we have studied the behaviour of mutants *cad2* and *pad2* [8, 9], affected in the glutathione biosynthesis pathway, as recently documented under TM and TMHT conditions [10]. In this chapter, we repeated those experiments and tested the survival of the mutants under three other stress regimes, namely Basal Thermotolerance (BT), Short-term Acquired Thermotolerance (SAT), and Long-term Acquired Thermotolerance (LAT). First, we obtained similar results as in Dard et al., with the low glutathione mutants *cad2* and *pad2* showing defects in TM (Figure 2) and TMHT (Figure 3), demonstrating the reproducibility of the method. Moreover, we show that *cad2* and *pad2* are also affected in BT, SAT, and LAT (Figure 4). As it is common in labs to include sucrose in plant growth media, we tested the effect of this variation on plant thermotolerance. We found that plants grown on media supplemented with 1% sucrose are generally more resistant to the stress regimes (Figure 4B-C). Consequently, the low survival phenotype of *cad2* and *pad2* in LAT (Figure 3) is not visible in the presence of sucrose (Figure 4C). Therefore, we recommend avoiding adding sucrose to the media for initial thermotolerance assays to prevent masking potential phenotypes.

2. Materials

2.1. Material references

Plant agar

2-(N-morpholino)ethanesulfonic acid (MES) hydrate

Murashige & Skoog medium + B5 vitamins

D(+) Sucrose

Round petri dish

Square petri dish

Plant growth chamber (SANYO MLR-351 or MLR-352)

Water bath

2.2. General considerations when performing thermotolerance assays

2.2.1. Plant age and daytime of the heat treatments:

BT, SAT, LAT and TMHT can be applied on 4 to 12 days post-germination. We observed that plant survival to heat stress is dependent on the age of the plants, generally decreasing while the treatment is applied on older plants (Dard et al., personal communications). Therefore, it is important to account for plant age when designing these experiments. This can be done as preliminary experiments by exposing wild-type plants at different ages (e.g. 4 to 12 days old) to BT, SAT, LAT and TMHT and evaluating their survival rate. 80-100% survival rate is generally accurate when expecting a mutant line to be more sensitive to heat stress than the wild-type. In case, a mutant line is expected to be more tolerant as wild-type, heat stress conditions affecting more severely the wild-type survival rate should be used.

Time of day influences plant thermotolerance, with *A. thaliana* Col-0 seedlings exhibiting varying survival rates depending on the duration of light exposure prior to the stress assay [11, 12]. Under

long-day conditions, maximum survival is observed after 8 hours of light, while the lowest survival occurs after 8 hours of darkness [12]. In this study, all stress assays were conducted after 4h of light exposure, where plants show moderate survival rate, when plants display moderate heat stress tolerance [12].

2.2.3. Heating device:

While TM and TMHT are relatively long-duration treatments (several days), high temperatures treatments in BT, SAT, and LAT are shorter (mins to hours). Thus, under these latter conditions, it is crucial to heat agar plates quickly. Therefore, for TM and TMHT, we use a preheated growth chamber (SANYO MLR-351 or MLR-352), while a preheated water bath is employed for BT, SAT, and LAT.

To evaluate the rate of temperature increase in both devices, we monitored temperature using a UT320D mini-contact dual-channel thermometer with two probes (one in the agar gel and the other in the air). The plates were either placed in a pre-warmed growth chamber (at 37°C) to simulate TMHT conditions, or in a water bath (at 44°C) to simulate BT, SAT, or LAT. In the water bath, the agar reached 44°C within 18 minutes, after which the plate was transferred to a recovery bath (20°C). Conversely, when using the growth chamber, the agar required more than 30 minutes to only reach 35°C. This rapid heating in the water bath is due to the high thermal conductivity of water, allowing a plate to reach 43°C in less than 5 minutes (Figure 5).

2.2.4. Internal controls:

We recommend adding WT plants as well as a mutant known to be sensitive to the stress regime. We use *hsf4aQK* and *hsp101* as control for TMHT and BT/SAT/LAT respectively [1, 13]. For TM, we generally use as control mutants *phyb* and *pif4*, which perform constitutive and impaired thermomorphogenesis, respectively [4, 5, 6].

3. Methods

3.1. Thermomorphogenesis:

1. Surface sterilize the seeds in 70% ethanol for 15 min, then wash with 100% ethanol and let them air dry.
2. While the seeds are drying, prepare the plates. In square plates, pour 50 ml of MS ½ without sucrose. Divide the plate vertically in two parts to grow two genotypes in parallel. Sow seeds with sterile toothpicks in a single row at the top of the square plate. Only 8 to 10 seeds per genotype can be sown (16-20 seeds in total per plate), to allow sufficient spacing for root and hypocotyl growth, and to facilitate measurement at the end.
3. Stratify the seeds for 2 days at 4°C then grow them vertically for 4 days at 20°C under long day conditions (16 h of light (120 W/m²) / 8 h of darkness).

Note 1: Stratification will synchronize the germination of imbibed seeds insuring an homogenous growth of the plants. While being stratified, it is likely that some of the seeds do not germinate or have a delayed germination. Therefore it is important to annotate these seeds before performing the heat stress. We have seen that plants germinated during or after the stress show a high resistance (leaves are green and plants grows quickly). Avoiding this step could give false results.

Note 2: Plates are placed vertically in the growth chamber to allow the measurement of roots and hypocotyls by the end of the thermomorphogenesis assay.

Note 3: At least 3 replicates of plates are made for the control condition (remaining at 20°C during the entire experiment) and the heat stress condition.

4. After 4 days, transfer seedlings to a plant growth chamber preheated at 27-28°C and grow the plants for 7 days under short-day conditions (8 h of light/16 h of darkness) for thermomorphogenesis assay.

Note 4: Arabidopsis plants show best hypocotyl elongation when grown under a combination of short day condition and warm temperature [14, 15].

5. After 7 days, open the plate, flatten the hypocotyls on the gel and take pictures of the plantlets from the top.

Note 5: Always place a ruler against the plate, to have a scale when measuring roots/hypocotyls on ImageJ.

6. Upload the picture on the ImageJ software [16] and measure root and hypocotyl lengths using the NeuronJ plugin available online <https://imagescience.org/meijering/software/neuronj/> [17].

Note 6: Pay attention to measure hypocotyls starting from the collet and ending between the cotyledons to avoid heterogeneity in the measuring.

3.2. Basal Thermotolerance (BT), Short-term Acquired Thermotolerance (SAT) and Long-term Acquired Thermotolerance (LAT):

1. Surface sterilize the seeds in 70% ethanol for 15 min, then wash with 100% ethanol and let them air dry.
2. While the seeds are drying, prepare the plates. In a round petri dish, pour 25 ml of MS $\frac{1}{2}$ with or without sucrose. Divide each plate into quarters using a pre-made pattern (if needed, more compartments can be included in each plate) and either spread or sow the seeds onto the MS $\frac{1}{2}$ medium with sterile toothpicks.

Note 7: Sowing with toothpicks makes it easier to calculate survival rates at the end of the experiment, and also results in neater plates.

Note 8: At least 3 replicates of plates are made for the control condition (remaining at 20°C during the entire experiment) and the heat stress condition.

3. Stratify the seeds for 2 days at 4°C then grow them for 5 days at 20°C under long day conditions (16 h of light (120 W/m²) / 8 h of darkness).

Note 1 also applies here.

4. After 5 days, the seedlings undergo BT, SAT or LAT stress. Tape the plate with electrician's tape (plasticized tape), wrap it in aluminum foil, and place in a watertight plastic bag. These steps prevent water from entering the plates, which would contaminate the medium and kill the plants. Place the plants in a pre-heated water bath to undergo the various stresses.
 - plates for BT: 30 min at 44°C
 - plates for SAT: 1h at 37°C then 2h recovery at 20°C then 30 min at 44°C
 - plates for LAT: 1h at 37°C then 2 days recovery at 20°C then 30 min at 44°C.

After each stress and before going to recovery, remove the plastic bag, aluminum foil and electrician tape.

Note 9: Wrapping plates with aluminium foil is advised to protect plants from light during the heat stress.

5. Following heat stress, transfer the plants at 20°C (16 h of light (120 W/m²) / 8 h of darkness) to recover for 7 to 10 days, then take pictures and calculate survival rates. Use 30 to 100 plants per replicate, scoring surviving plants based on their ability to produce new green leaves after recovery.

Note 10: Determine if a plant is dead or not can be challenging. Some plants can look very affected with bleached leaves but still be able to produce new leaves. An early indication that the plant is alive is the meristem that is still green and is likely to make new leaves.

3.3. Thermotolerance to moderately high temperature (TMHT):

1. Surface sterilize the seeds in 70% ethanol for 15 min, then wash with 100% ethanol and let them air dry.
2. While the seeds are drying, prepare the plates. In a round petri dish, pour 25 ml of Murashige and Skoog (MS) ½ medium (MS (2.2 g/L), MES 0.5%, plant agar 0.8%, pH adjusted to 5.8 with 1 N KOH). Divide each plate into quarters using a pre-made pattern (if needed, more compartments can be included in each plate) and either spread or sow the seeds onto the MS ½ medium with sterile toothpicks.

Notes 7 and 8 also apply here.

3. Stratify the seeds for 2 days at 4°C then grow them for 5 days at 20°C under long day conditions (16 h of light (120 W/m²) / 8 h of darkness).

Note 1 also applies here.

4. After 5 days, transfer the seedlings to a plant growth chamber preheated at 37°C for 5 to 7 days under long day conditions for the thermotolerance assay.
5. Following heat stress, transfer the plants at 20°C to recover for 7 to 10 days, then take pictures and calculate survival rates. Use 30 to 100 plants per replicate, scoring surviving plants based on their ability to produce new green leaves after recovery.

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Author Contributions

Writing, review and editing, JA, JPR and AD; All authors have read and agree with the present version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends:

Figure 1: Different high temperature regimes to study heat stress responses in plants. (A) Thermomorphogenesis. (B) Basal thermotolerance (BT). (C) Short-/long-term acquired thermotolerance (SAT/LAT). (D) Thermotolerance to moderately high temperature (TMHT). See text for more details on the conditions of the respective treatments.

Figure 2: Low glutathione impairs thermomorphogenesis. (A) Pictures of representative Col-0, *cad2* and *pad2* plants taken after 11 d of treatment. Black bar=10 mm. (B) Hypocotyl length measurements.

Figure 3: Low glutathione impairs thermotolerance to moderate high temperature. Wild-type plants (Col-0), *cad2*, *pad2*, and the Heat Shock Factor A1 quadruple knockout mutant (*hsfa1qk*) were subjected to a (TMHT) regime. (A) Pictures of the plates grown at 20°C (left) or subjected to 37°C (right) were taken 10 d after recovery. (B) The viability of the plants is assessed by the recovery of shoot growth.

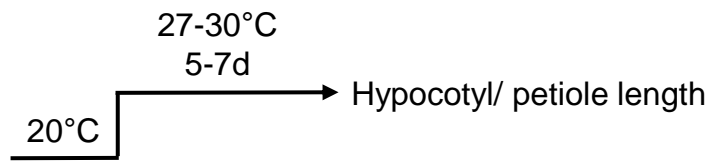
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Figure 5: Study of temperature rise time during different heat treatments. Temperature was monitored using a UT320D mini-contact dual-channel thermometer with two probes (one in the agar gel and the other in the air). The device was placed in a pre-warmed growth chamber (at 37°C) to

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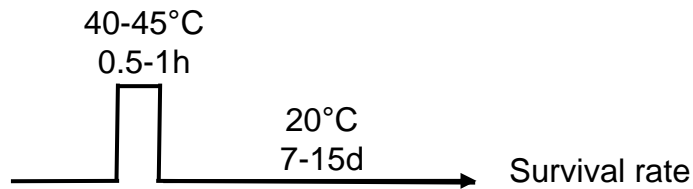
A

Thermomorphogenesis



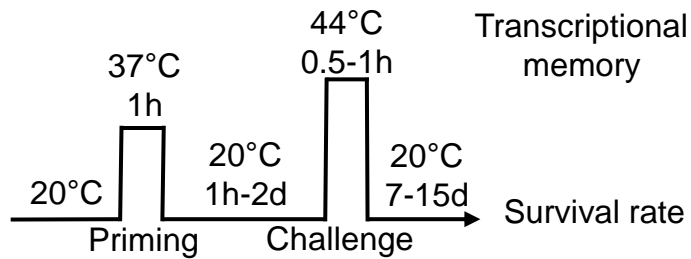
B

BT (Basal Thermotolerance)



C

SAT/LAT (Short/Long-term Acquired Thermotolerance)



D

TMHT (Thermotolerance to Moderately High Temperature)

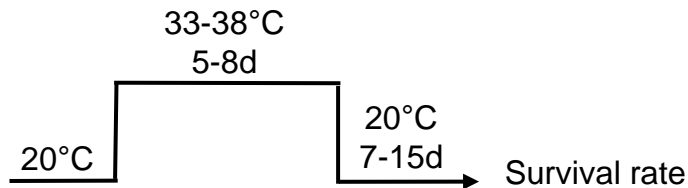


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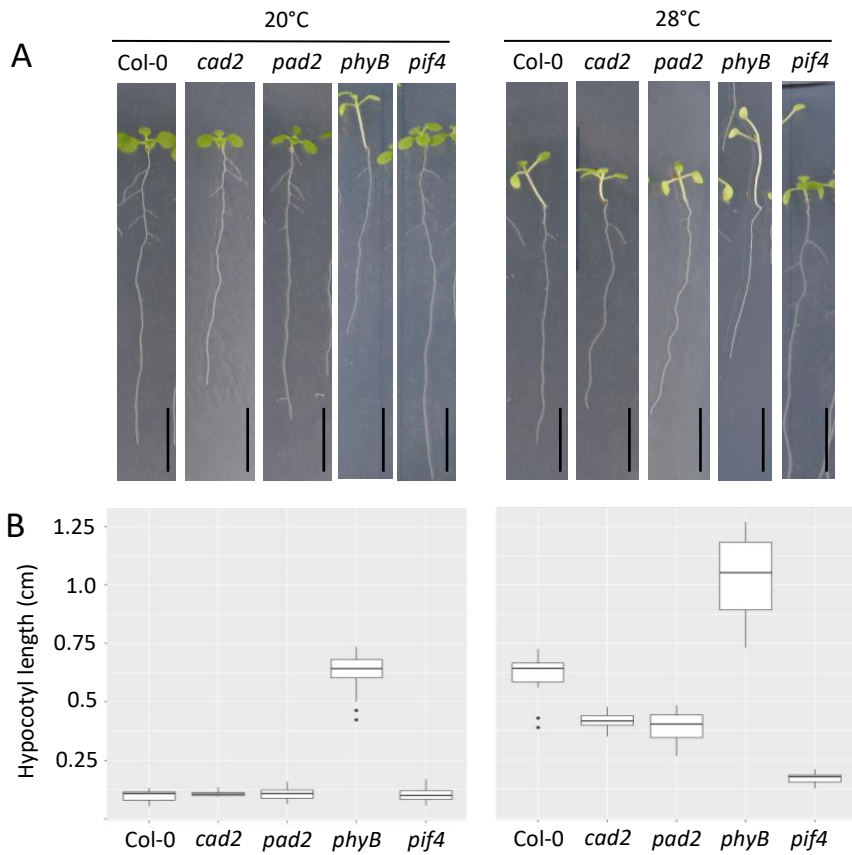


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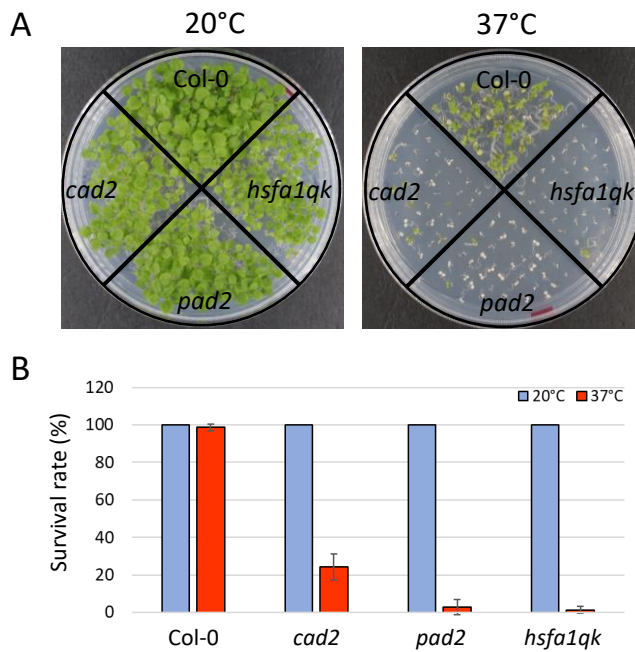


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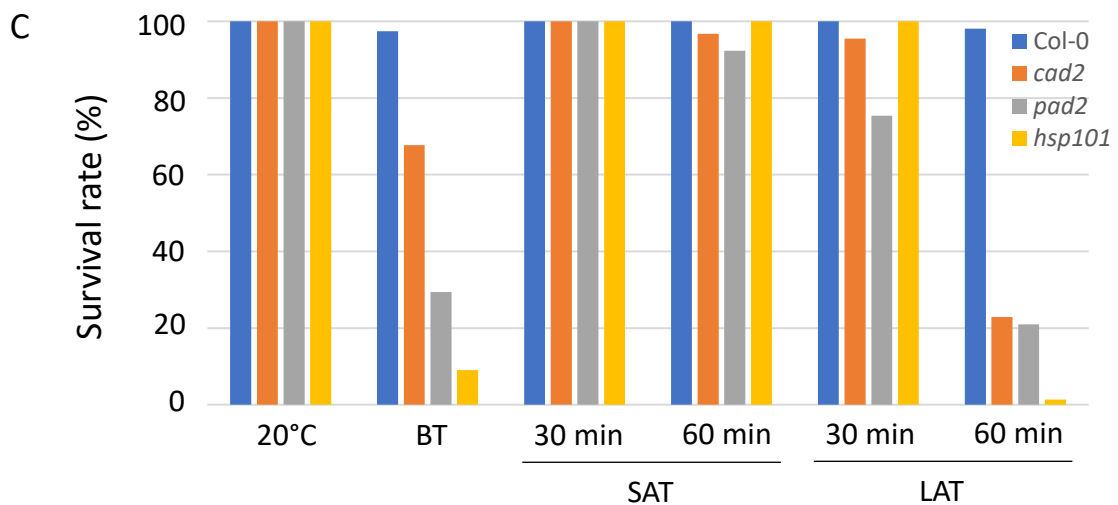
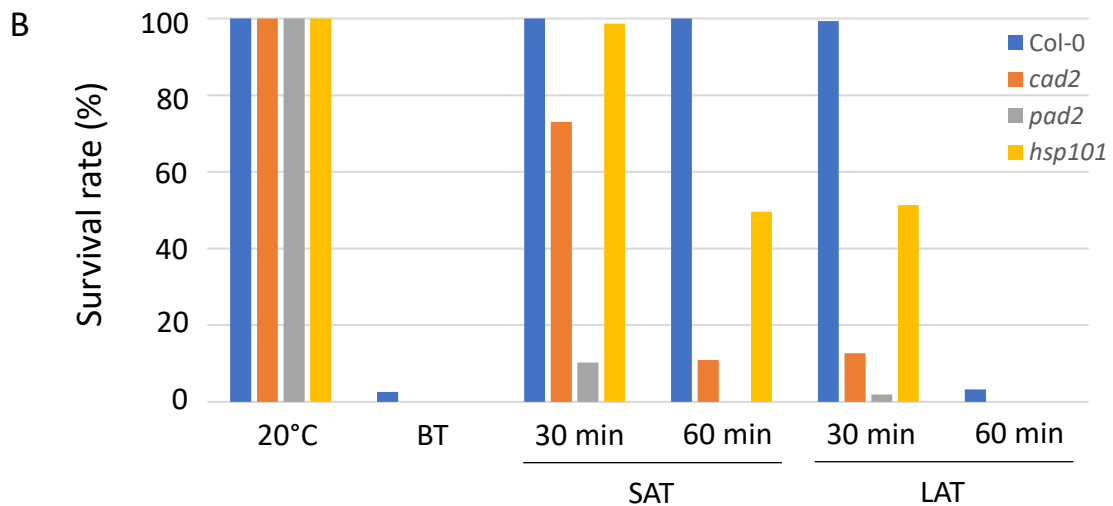
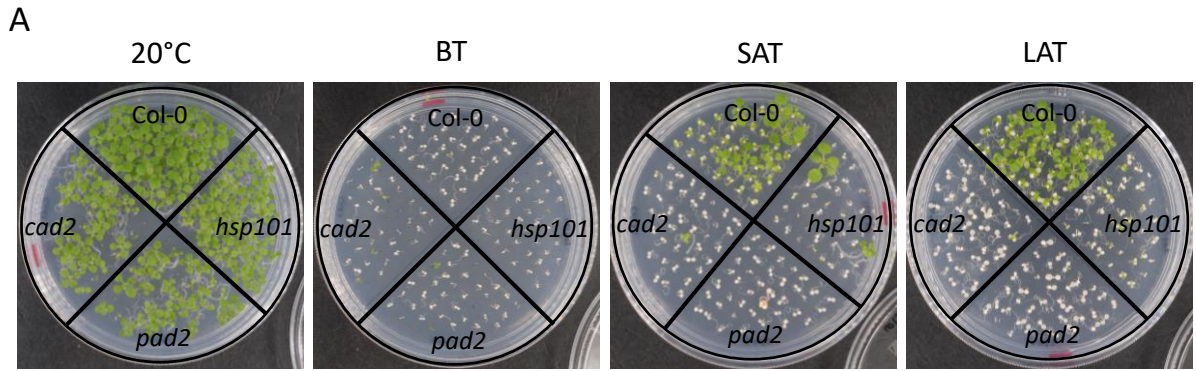


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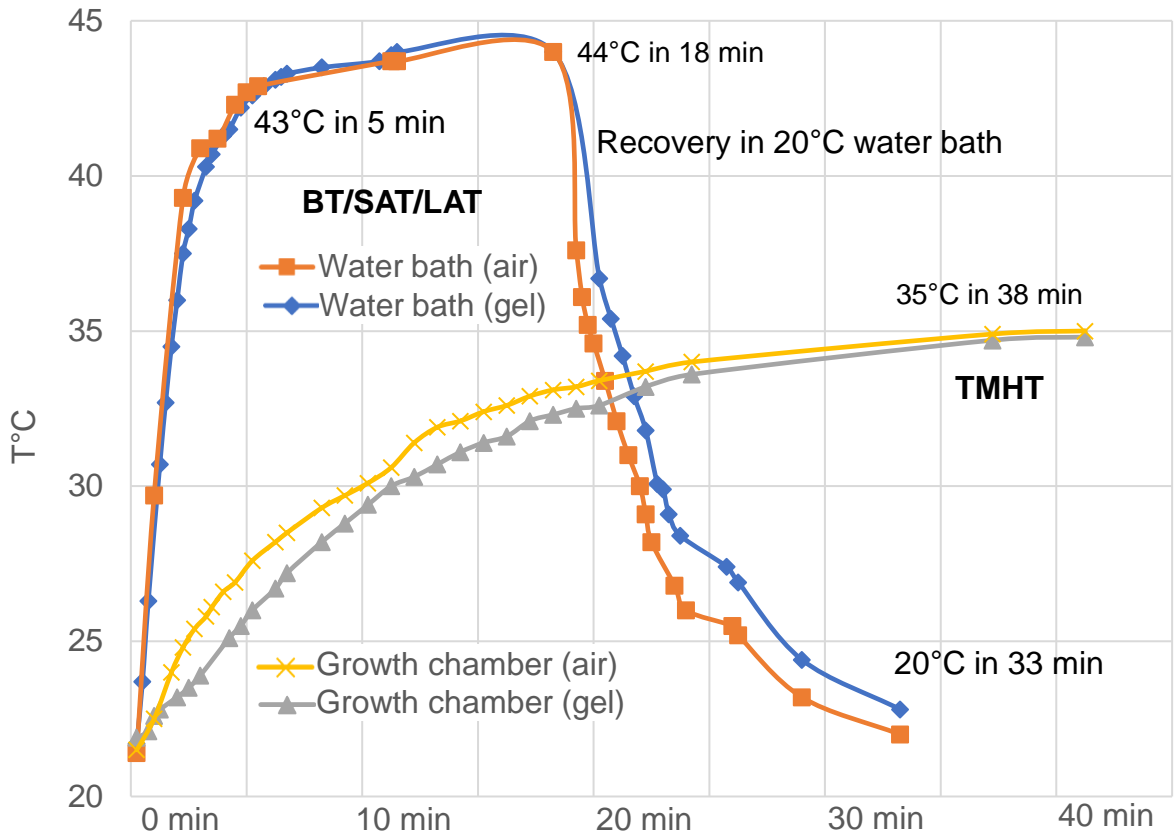


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