IRE1 regulated autophagy and chaperone levels cooperatively modulate acquired thermotolerance in *Arabidopsis thaliana*

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Short title: IRE1 regulates autophagy during priming

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Abstract

Rapidly changing global climatic conditions emphasize the urgent need for the development of heat stress-resilient plants. Priming, a stress-training program that aids in imparting tolerance to subsequent stress(es) emerged as a promising approach to improve stress tolerance. However, molecular mechanisms underlying priming-induced plant stress responses still remain largely elusive. In the current study, the impact of heat stress on *Arabidopsis thaliana* seedlings pre-exposed to elevated temperature (primed) was found to be minimal, compared to naïve (unprimed) seedlings, which failed to survive. Our findings suggest that thermopriming-mediated heat stress tolerance operates through the activation of IRE1-bZIP60 cascade triggering unfolded protein response (UPR). Further, IRE1 activation triggers autophagy response through attenuation of autophagy-negative regulators such as *BGLU21, ROSY1* and *PR-14* via RIDD-mediated mRNA degradation. Alongside UPR and autophagy, priming temporally induces HSP levels, rendering plants tolerant to acute heat stress. Contrarily, unprimed seedlings fail to mount such temporally coordinated regulation, thus fail to survive. Moreover, loss of bZIP60 reduces the impact of priming-induced response, suggesting its role in the maintenance of stress memory. Taken together, the current study suggests that priming-mediated regulation of protein homeostasis through the temporal regulation of autophagy in concert with chaperone synthesis promotes heat stress tolerance.

Keywords

Priming, thermotolerance, autophagy, ER stress, unfolded protein response, RIDD (Regulated IRE1-dependent decay of mRNA)

Introduction

The upsurge in human population has led to an increase in the food demand affecting the Global Food Security Index (GFSI). Recent GFSI statistics indicate a serious threat to the food security, and demands immediate attention to improve food production in terms of both quality and quantity (GFSI 2022). This has further augmented the research efforts for up-scaling food production. However, in nature, plants often encounter variable abiotic and biotic stresses, affecting their productivity. High temperature is one such abiotic stressor which is detrimental to the plant growth, productivity or even survival. Studies have reported that for every 2°C rise in temperature, there could be a 50% decline in crop productivity (Lobell et al., 2012). Heat stress reduces the protein, starch, and oil content thereby limiting plant growth, development, as well as biomass (Fahad et al., 2017). To combat heat stress-related deleterious effects, researchers tried to overexpress heat shock proteins (HSPs) in plants (Nowicka et al., 2018); however, it is observed that overexpression of HSPs itself acts as stress, thereby resulting in unintended damaging effects (Nouri & Komatsu, 2013).

Interestingly, recent studies identified pre-exposure to a stress stimulus (priming) to dramatically improve plant stress tolerance. 'Priming' is a memory based physiological response in which an organism mounts a heightened defense response to biotic and/or abiotic stresses (Conrath, 2011; Conrath et al., 2007; Wilkinson et al., 2019). Primed state of an organism can be induced by either natural or artificial stimuli. These stimuli can remodel the cells/organism at genetic, epigenetic, and metabolic levels, increasing the organism's robustness against subsequent stress (Catoni et al., 2022; Espinas et al., 2016; Gamir et al., 2014; Serrano et al., 2019; Tugizimana et al., 2018). Priming-mediated acquired stress tolerance has been previously studied in model plant system *Arabidopsis thaliana* and several crop plants (Balmer et al., 2018; Liu et al., 2021; Samantaray et al., 2023; Song et al., 2015; Westman et al., 2019; Ye et al., 2019).

Previous studies identified priming to induce various chromatin remodelers such as histone-acetylases, -methyltransferases and -demethylases, leading to acetylation and methylation of histones (H3K4/H3K9/H3K2 trimethylation) as well as methylation of DNA, imparting stress tolerance (Lämke et al., 2016; Nair et al., 2022). These epigenetic modifications aid in inducing the expression of several stress-responsive genes such as *HSFA2*, sHSPs (*HSP21, HSP22, HSP18.2*) in response to heat-stress (Lämke et al., 2016); *RAB18* and *RD29B* in response to drought stress (Ding et al., 2012); *P5SC1* in response to salt stress (Buzas, 2017; Feng et al., 2016); *COR* genes, *WRKY19* and *IR12* in

87 response to cold stress (Thalhammer et al., 2014; Wang et al., 2021), etc. While several studies
88 focused on understanding the epigenetic regulation underlying priming response (Crespo-Salvador et
89 al., 2018; Kotkar & Giri, 2020; Noh et al., 2021; Turgut-Kara et al., 2020), very little is known about
90 the role of post-transcriptional processes such as protein homeostasis machinery, autophagy,
91 mitochondrial dynamics. We surmised that since heat-stress is likely to involve disruption of protein
92 homeostasis, we probed the underlying effects, if any, by monitoring protein homeostasis maintenance
93 pathways - chaperone synthesis and autophagy during priming.

94 Autophagy is a process that helps in the degradation of damaged organelles (Janse van Rensburg et
95 al., 2019) and promotes the recycling of dysfunctional proteins/aggregates during heat-stress (Su et
96 al., 2020; Zhou et al., 2013). Autophagy is regulated by three major signaling kinases – TOR, SnRK,
97 and IRE1 (**Supplementary Figure 1**). The Target of rapamycin (TOR) protein functions as a complex
98 and gets activated in energy-rich conditions. Upon activation, it phosphorylates ATG13 and ATG1
99 proteins and inhibits the activation of the autophagosome initiator complex, thus inhibiting autophagy
100 (Suttangkakul et al., 2011). Contrarily, the sucrose non-fermenting 1 (SNF1)-related protein kinase
101 (SnRK) gets activated in energy-depleted conditions. Upon activation, it phosphorylates the RAPTOR
102 protein of the TOR complex which inhibits TOR complex formation, thus rendering autophagy to be
103 active (Soto-Burgos & Bassham, 2017). SnRK is also speculated to activate autophagy directly, but
104 the underlying mechanism is less understood (Janse van Rensburg et al., 2019). Inositol-requiring 1
105 (IRE1) is an endoplasmic reticulum (ER) membrane protein that is activated upon ER stress (Nawkar
106 et al., 2018; Pastor-Cantizano et al., 2020) and is speculated to regulate autophagy through its mRNA
107 degradation activity (Bao et al., 2018). Autophagy has also been identified to reset heat-stress memory
108 through the degradation of various HSPs such as HSP17.6, HSP21, HSP90 and HSP101 during stress
109 recovery period (Sedaghatmehr et al., 2019, 2021; Thirumalaikumar et al., 2020). While autophagy
110 has been linked to the degradation of HSP21 and HSP90.1 involving a metalloprotease FtsH6 and
111 NBR1 (next to BRCA1 gene 1), mediated mechanisms, respectively (Sedaghatmehr et al., 2021;
112 Thirumalaikumar et al., 2020). However, how autophagy and the associated heat shock protein
113 response is regulated during priming, remains largely elusive.

114 Gaining insights into the regulation of autophagy would allow us to comprehend the molecular
115 dynamics governing heat stress memory. In the current study, we observed that thermopriming leads
116 to temporally regulated autophagy that acts in coordination with chaperone induction, promoting

survival against severe heat-stress. Upon priming, the seedlings survive and appear nearly as good as the unstressed controls, whereas the unprimed seedlings die off. Upon heat stress, IRE1 gets activated in primed seedlings, triggering unfolded protein response (UPR) through the activation of bZIP60 splicing activity. In parallel, IRE1 activation also selectively degrades mRNA of autophagy-negative regulators, thereby triggering autophagy response. Most importantly, thermopriming renders timely coordination between chaperone synthesis/UPR and autophagy regulation leading to efficient and timely removal of protein stress, causing heat tolerance. Contrarily, unprimed plants do not exhibit such concerted regulation of UPR and autophagy, thus fail to survive.

Methods

Plant materials and growth conditions

Arabidopsis thaliana (Col-0) seeds were surface sterilized by washing with 80% ethanol for 30 seconds (s) followed by 1% bleach for 3 minutes (min), and 3x washes with sterile double distilled water. Seeds were then sown on ½ Murashige and Skoog media with 1% sucrose and 0.8% agar powder. The seeds were stratified at 4°C for two days before transferring them to growth chambers with 16 hour (h) light and 8h dark, 65% humidity. The 5-day-old *Arabidopsis* seedings were primed by incubating them in a water bath at 37°C for 90 min, followed by a recovery at 22°C for 90 min and heat-triggering at 45°C for 45 min. The unprimed samples were directly incubated at 45°C for 45 min. For primed-control condition, seedlings were only subjected to a 37°C treatment for 90 min and were returned to optimal growth conditions. Control seedlings were maintained at optimal growth conditions (22°C, 16h light and 8h dark, 65% humidity) all through the experiment. Samples were collected at different stress recovery time points for protein and gene expression analysis. The treatment conditions described above were also used for the analysis of *bzip60* (CS813074) and ER-GFP (CS16251) lines obtained from ABRC (Arabidopsis Biological Resource Center).

Phenotypic analysis

Post exposure to priming or triggering heat stress, seedlings were analyzed for leaf number, root length, and rosette area to assess growth. Leaf number and root length were measured manually using the image J software. For rosette area measurement, the thresholding method on ImageJ software was

used. The images of seedlings were first converted to 8 bits, followed by adjusting the threshold to reveal seedlings in the form of a defined area. The images were then converted to binary, and the area was measured using ImageJ tools. The measurements were taken for phenotypic analysis where, n = number of individual seedlings. The data was plotted as mean for root length (n=5/6), leaf number (n=32), and rosette area (n=16) using GraphPad software. The experiments were conducted using two biological replicates.

Protein extraction and Western blot analysis

Soluble protein was extracted from 5-day-old *Arabidopsis* seedlings as previously described in (Yamamoto et al., 2018) with a few modifications. About 40-50 mg of seedlings (n=16) were collected and frozen in liquid nitrogen. The samples were ground using Retsch Miller MM400 at 25Hz for 30s (x2 cycles). After grinding, 200-300µl of 1x RIPA lysis buffer (Merck; 20-188) containing 10% glycerol, 1x phosphatase inhibitor cocktail (PhosSTOP, Roche; 4906837001), and 1x protease inhibitor cocktail (Roche; 4693132001) was added and incubated at 4°C for 1h at 1500 rpm. The samples were centrifuged at 14000 rpm for 30 min at 4°C, after which the supernatant containing protein was collected and stored at -80°C until usage.

For protein analysis, protein concentration was estimated using BCA method, following the manufacturers' instructions (Cat. No. 23225). Protein (20 µg per lane) was loaded onto 8% SDS-PAGE gel, which was stained using Coomassie Brilliant Blue. Protein bands were visualized after destaining the gel following a standard protocol (CSH protocol, 2007). For Western blot analysis, 20 µg of protein was loaded onto 8-12% SDS-PAGE, following which the proteins were transferred to the nitrocellulose membrane. The blot was blocked using 5% BSA or 2% skim milk solution for 1h at room temperature (RT), followed by primary antibody incubation (overnight at 4°C). The blot was developed after secondary antibody incubation (1h at RT) using Immobilon Forte HRP substrate (Cat. No. WBLUF0500). α-Tub (CST 3873), ATG8 (Abcam, ab77003), HSP70 (Abcam, ab2787), HSP90 (Sigma, CA1023), HSP17.6 (Agrisera, AS07 254), HSP21 (Agrisera, AS08 285), HSP101 (Agrisera, AS08 287), pS6K (CST 9234), S6K (CST 9202) and pAMPK-α (CST 2535) were used as primary antibodies. Anti-rabbit (sc-2313) and anti-mouse (CST 7076) were used as secondary antibodies. The experiments were conducted using three biological replicates.

Gene Expression Analysis

For gene expression analysis, 5-day-old *Arabidopsis* seedlings were collected at several time points (post priming, triggering and during recovery) and frozen in liquid nitrogen. The samples were ground using Retsch Miller MM400 at 25Hz for 30s (x2 cycles). After grinding, RNA was isolated from the samples using a combination of TRIzol/chloroform extraction and kit method (PureLink™ RNA Mini Kit, Thermo; 12183018A). The purified RNA was treated with DNase I (NEB; M0303) following standard protocol. cDNA was synthesized using 1 µg of RNA following manufacturers' instruction (1st strand cDNA synthesis kit, Takara; 6110A). RT-PCR was performed using SapphireAmp® Fast PCR Master Mix (Takara; RR350A), while iTaq™ Universal SYBR® Green Supermix (Biorad; 1725121) was used for performing qRT-PCR. The experiments were conducted using three biological replicates. Gene accession IDs and PCR primer details are described in **Supplementary Table 1 and 2**, respectively.

Microscopy

After priming, the ER-GFP seedlings were collected post heat stress, and fixed using 4% paraformaldehyde solution (Himedia; TCL119) containing 0.1% Triton X-100 (Zhu et al., 2020). After adding the fixating solution, the samples were kept in a desiccator for 30-40s after applying the vacuum. The process was repeated several times until the explants sunk. The samples were then incubated with shaking in fixating solution for 20 min, followed by wash with 1xPBS (3 times) for 5 min. The seedlings were then transferred to a glass slide and placed under a coverslip. *Arabidopsis* leaf tissue was visualized under 40x magnification using Zeiss LSM 710 confocal microscope. A total of 16 seedlings from four independent experiments were imaged for each time point.

Statistical Analysis

The data was plotted using GraphPad Prism 8.0. The data was analyzed using One-way ANOVA (for qPCR data), Two-way ANOVA and t-test (for phenotype analysis) to obtain statistics. p values <0.05 were considered as significant (**Supplementary Data 1-3**).

Supplementary Information

Supplementary Figure 1: Autophagy regulatory network

Supplementary Figure 2: TOR and SnRK activity do not change during priming

Supplementary Figure 3: ER-GFP signal in leaf samples post-stress

Supplementary Figure 4: Identification of homozygous knockout *bzip60* mutant lines by genotyping analysis

Supplementary Figure 5: Primed wild-type seedlings exhibit better survival than unprimed seedlings upon heat-stress

Supplementary Figure 6: *bzip60* mutants exhibit slower recovery in rosette growth than wild-type post priming

Supplementary Figure 7: Model for IRE1 activation during priming

Supplementary Table 1: List of gene accession IDs

Supplementary Table 2: List of PCR primers

Supplementary Data 1: Statistical information of phenotypic analysis

Supplementary Data 2: Statistical information of qPCR analysis

Supplementary Data 3: Statistical information of western blotting analysis

Results

Priming improves survival from severe heat-stress

To understand the mechanism that drives thermopriming-mediated heat stress tolerance, we exposed the five-day-old Arabidopsis Col-0 seedlings to 37°C (for 90 min), allowed them to briefly recover at optimum conditions (for 90 min) before exposing them to triggering heat stress (45°C for 45 min) (**Figure 1A**). Hereafter, this condition is referred to as 'primed'. Effect of thermopriming was analyzed by comparing the impact of lethal heat stress on primed *vs.* naive (unprimed: directly exposed to triggering heat stress) seedlings. Post heat stress, we recorded the seedling growth as a function of time under all the conditions tested (control, primed control, primed and unprimed). Primed seedlings did not display root growth immediately post exposure to heat stimuli. However, they resumed quickly and displayed growth similar to control seedlings, which was quantified as

226 increase in leaf number and root length (**Figure 1, B-D**). In contrast, the unprimed seedlings showed
227 arrested growth, with no increase in leaf number and root length (**Figure 1, B-D; Supplementary**
228 **Data 1**). Moreover, by 5 days post heat stress, the unprimed seedlings display visibly complete loss of
229 chlorophyll, and fail to survive (**Figure 1B**). To summarize, heat stress affects growth in both primed
230 and unprimed conditions. However, thermopriming abrogates the lethal effects of heat stress, and aids
231 in quickly resuming growth in the primed seedlings.

232 **Thermopriming mitigates heat stress-induced protein damage**

233 Heat stress may damage labile macromolecular complexes affecting several biological processes,
234 thereby threatening plant survival (Kotak et al., 2007; Mittler et al., 2012; Zhao et al., 2020).
235 Importantly, heat stress affects the structure and functionality of proteins (Mogk et al., 2018), which are
236 majorly thermolabile. Notably, the misfolded proteins either form aggregates (McLoughlin et al., 2019;
237 Mogk et al., 2018) or get degraded, in the absence of robust chaperone activity (Hartl et al., 2011). In
238 order to understand if post heat stress seedling survival is associated with the efficient maintenance of
239 protein pool, we first analysed the total protein content in the primed, unprimed and control seedlings
240 at multiple time points (**Figure 2A**). The protein content in the control (grown at optimal conditions at
241 22°C), primed control (subjected to only priming stimuli at 37ºC and returned to optimal conditions)
242 and primed seedlings is found to be similar until day 3, post heat stress (**Figure 2B**). On the other hand,
243 unprimed samples display a consistent drop in total protein levels from day 1 to day 3 post heat stress,
244 suggesting attenuated chaperone activity, resulting in protein degradation (**Figure 2B**). These
245 observations are consistent with the previous reports showing that protein content reduces upon
246 exposure to noxious heat stress (Gulen & Eris, 2004; Nagy-Réder et al., 2022). Our results suggest that
247 priming-mediated heat stress tolerance involves efficient maintenance of protein homeostasis.

248 **Priming modulates proteostasis through temporal regulation of autophagy and chaperones**

249 We next probed if priming-mediated protein homeostasis involves autophagy. Autophagy is a process
250 which aids in the clearance of misfolded/damaged proteins or their aggregates that form during stress
251 (Avin-Wittenberg, 2019; Hartl et al., 2011; Liao & Bassham, 2020). We used ATG8 as a marker to
252 quantify autophagy response post heat stress in a time-course manner, across all the conditions. ATG8
253 is an autophagosome membrane protein crucial for autophagosome formation (Shpilka et al., 2011),
254 and elevated ATG8 levels correlate with increased autophagy (Upadhyaya et al., 2020). In order to

255 determine if exposure to priming stimulus itself can trigger autophagy, we checked the ATG8 levels
256 immediately after priming (AP). We observed that both control and primed seedlings display similar
257 levels of ATG8 (**Figure 2C**). However, post exposure to triggering heat stress (Day 0), ATG8 levels
258 increase in both unprimed and primed seedlings compared to control (**Figure 2C**). Interestingly, such
259 an increase in ATG8 level was more pronounced in the primed seedlings (**Figure 2C**). Further, to
260 assess if the heat stress-induced autophagy sustains or declines, or surges with time, we determined
261 ATG8 levels from 1 to 3 days post heat stress. In the unprimed seedlings, the ATG8 levels increased
262 further by Day 1, which continued until Day 2 (**Figure 2D**). Notably, the visible decline in ATG8
263 levels (in Day 3) correlate with the reduced protein content in the unprimed seedlings (**Figure 2B**).
264 Contrarily, primed seedlings show reduced ATG8 levels by Day 1 (compared to Day 0), and the levels
265 are similar to that of control seedlings (**Figure 2D**). No difference in ATG8 levels were detected in
266 primed control compared to control (**Figure 2D**). An increase and decrease in ATG8 levels in the
267 unprimed *vs.* primed, respectively, by Day 1, suggests dynamic regulation of autophagy in the primed
268 seedlings between Day 0 to Day 1, post heat stress. To understand this, we analyzed the ATG8 levels at
269 hourly intervals post heat stress (**Figure 2E**). As shown in **Figure 2F**, in primed, ATG8 levels peak by
270 0h, which gradually drop by 6-8h to a level similar to that of control seedlings. In contrast, unprimed
271 seedlings display a peak in ATG8 levels by 2h, that stays high much longer beyond 8h, and until Day 2
272 (**Figure 2, D and F**). Such decrease or increase/sustenance of autophagy in the primed *vs.* unprimed,
273 respectively, suggests priming-mediated robust activation of protective mechanisms, and may involve
274 chaperons, to maintain protein homeostaisis post heat stress.

275 In order to test this hypothesis, we next analyzed chaperone levels (HSP70 and HSP90) post heat stress
276 across all conditions. Exposure to priming stimulus (AP) results in increased accumulation of HSP70
277 and HSP90, which sustain until Day 1 post exposure to triggering heat stress, in the primed compared
278 to control (**Figure 3, A and B**). However, the unprimed fail to mount such response with the HSPs
279 abundance being lower than the control seedlings (**Figure 3, A and B**). In addition, we analyzed the
280 abundance of thermomemory-related chaperones HSP17.6, HSP21 and HSP101 (Bäurle, 2016; Bäurle
281 & Trindade, 2020; Khan et al., 2022) at all the times points stated above. As expected, their levels were
282 higher in the primed compared to unprimed and control, post exposure to priming/triggering heat stress
283 (**Figure 3, C and D**). Immediately upon exposure to priming (AP timepoint), increased abundance of
284 HSP21 and HSP101 was detected (**Figure 3C**), while the HSP101 and HSP17.6 are retained longer in
285 the primed control (**Figure 3D**). Increased levels of all the three memory HSPs was detected in the

primed, but not in the unprimed or control seedlings (**Figure 3, C and D**).

Furthermore, in order to assess whether the HSP abundance described thus far parallel transcript levels across all conditions, we profiled the *HSPs* expression using qPCR (**Figure 3, E-I**). As expected, in primed seedlings, abundance of HSPs correlated with their elevated transcript levels compared to that of control. Surprisingly, unprimed seedlings displayed gradual increase in *HSPs* transcript abundance post heat stress (**Figure 3, E-I**) which does not correlate with the protein levels observed (**Figure 3, A-D**). This suggests that either post-transcriptional RNA processing or translation itself is attenuated in the unprimed samples. Nonetheless, primed-controls display increased *HSPs* abundance which gradually declines with time (**Figure 3, E-I**). These results suggest that priming aids in timely activation of autophagy and chaperone response to maintain protein homeostasis, thereby enhance plant heat stress tolerance. In contrast, unprimed seedlings display sustained autophagy with very low to undetectable chaperone levels and fail to survive.

Regulation of autophagy under heat stress is TOR and SnRK independent

Previous studies have identified the role of TOR and SnRK in the regulation of autophagy under stress (Liao & Bassham, 2020; Pu et al., 2017; Soto-Burgos & Bassham, 2017; Yang et al., 2023). We wondered if TOR/SnRK are involved in the priming-mediated regulation of autophagy in response to heat stress. In order to test this, we analyzed the phospho-S6K (pS6K) levels as a measure of TOR kinase activity (Raught et al., 2001; Upadhyaya et al., 2020) and active-SnRK (pAMPK: using phospho-AMPKα antibody) for SnRK activity (Baena-González et al., 2007; Belda-Palazón et al., 2020). Interestingly, neither pS6K nor the pAMPK levels were significantly altered at all the time points across all the conditions **(Figure 4, A-D; Supplementary Figure 2**). These findings suggest that priming- and heat stress-mediated regulation of autophagy (as observed in **Figure 2, C-F**) is independent of TOR and SnRK.

Unprimed samples exhibit sustained ER stress

We next checked if the abrogated chaperone activity in the unprimed seedlings is associated with the endoplasmic reticulum (ER) stress, as dysregulation in protein homeostasis is known to impact ER functional status (Humbert et al., 2012). IRE1 is an ER membrane protein, involved in the maintenance of protein homeostasis in the ER (Nawkar et al., 2018; Pastor-Cantizano et al., 2020). Upon stress, IRE1 activation leads to splicing of *bZIP60*, forming *bZIP60* spliced form (*bZIP60s*),

which is known to transcriptionally modulate genes involved in unfolded protein response (Bao et al., 2018; Deng et al., 2011; Humbert et al., 2012; Ruberti et al., 2018). In order to examine the ER stress levels, if any, we analyzed the transcript abundance of *bZIP60s* across all the conditions at different time points post priming/triggering stress. Interestingly, exposure to priming temperature itself triggered ER stress (*bZIP60s* form visible at AP; **Figure 5A**). Post triggering heat stress, although primed seedlings display ER stress initially (AT/Day 0), they recover by Day 1 (**Figure 5A**). In contrast, the unprimed seedlings display elevated and sustained *bZIP60s* levels post exposure to triggering heat stress, suggesting high ER stress compared to control (*bZIP60s* undetectable).

In addition, to monitor the ER status visually across different conditions, we imaged the ER using an ER specific GFP marker (Nelson et al., 2007). In the unprimed, although GFP signal was visible immediately post triggering heat stress (Day 0), it dramatically reduced to undetectable levels by Day 1-3 (**Figure 5B; Supplementary Figure 3**). Contrarily, the primed seedlings display ER-GFP signal similar to that of the control, at all the time points tested. These results suggest that priming aids in the efficient maintenance of ER under heat stress, which is absent in the unprimed, resulting in the disruption of the ER, leading to seedling death. Taken together these findings indicate that thermopriming mitigates the heat stress-induced ER stress, promoting seedling survival.

ER associated IRE1 protein modulates autophagy during priming

In addition to regulating *bZIP60* splicing, IRE1 also modulates autophagy by fine-tuning the RIDD (Regulated IRE1-dependent decay of mRNA) activity (Li & Howell, 2021; Maurel et al., 2014), which in turn leads to the degradation of mRNA (Bao et al., 2018; Chen & Brandizzi, 2013; Srivastava et al., 2018) . Notably, RIDD leads to the downregulation of inhibitors of autophagy such as *BGLU21*, *ROSY1* and *PR-14,* by targeting those mRNAs for degradation (Bao et al., 2018). We surmised that reversal to basal level *vs.* more sustained autophagy in the primed and unprimed, respectively, may involve RIDD-mediated regulation. Therefore, we probed *BGLU21*, *ROSY1* and *PR-14* transcript levels across all the conditions and time-points tested. At the time points- post priming (AP), before triggering (BT) and after triggering (AT: Day 0), primed seedlings showed significant reduction in the transcript levels of all the three genes compared to control (**Figure 5, C-E**). However, by Day 1 to 2 post triggering heat stress, we observed a recovery in their transcript abundance (**Figure 5, C-E**). Opposite to that, the transcript levels of *BGLU21* and *PR14* significantly remained lower in unprimed seedlings at all the time points compared to control (**Figure 5, C-E**).

Whereas, except *BGLU21* at after triggering stress time-point, no significant difference in transcript abundance was observed in the primed control compared to control seedlings (**Figure 5, C-E**). Particularly, these changes in the transcript abundance correlate well with the ATG8 levels in these seedlings (**Figure 2, C-F**). For instance, in the primed, reduced transcript abundance of these autophagy inhibitors correlates with the induced autophagy (at AP, BT, AT). At later time points post triggering (Day 1-2), when the transcript abundance of these inhibitors reverts to near control level, autophagy was found to return to basal level in the primed (**Figure 2, C-F; Figure 5, C-E**). Contrarily, sustained induction of autophagy aligns with reduced transcript abundance of the autophagy inhibitors in the unprimed, post exposure to triggering heat stress (**Figure 2, C-F; Figure 5, C-E**). These observations suggest that priming modulates ER status through IRE1-RIDD-mediated regulation of autophagy.

Loss of bZIP60 leads to delay in priming-mediated heat stress-recovery

As our results indicate that priming-mediated regulation of plant response to heat stress involves IRE1-bZIP60 and the associated modulation of ER status, we wanted to check whether loss of bZIP60 has any impact on the priming-induced stress responses outcomes. We tested the paradigm of priming with *bzip60* knockout mutants (**Supplementary Figure 4**) compared to wild-type (WT). When directly exposed to triggering heat stress (unprimed), both *bzip60* and the WT seedlings failed to survive (**Figure 6A; Supplementary Figure 5**). Interestingly, the positive effect of priming on seedling growth upon heat stress was also observed in the *bzip60* seedlings albeit to a lower level compared to WT (**Figure 6**). Further, the growth rate of primed seedlings is found to be slower in the *bzip60* compared to WT (**Figure 6, B and C; Supplementary Figure 6**). Collectively, 7 days post heat stress the root length was found to be significantly lower in the *bzip60* primed compared to control, while the root length of primed was found to be similar to that of control in WT (**Figure 6, D-F**).

Further, to gain insights in to the regulation of priming-mediated response to heat stress in the *bzip60*, we analyzed the chaperone levels post exposure to triggering heat stress in the primed, unprimed and control *bzip60* seedlings. Primed *bzip60* seedlings display elevated chaperone levels from Day 0 to 2, which subsided by Day 3 (**Figure 7**), opposite to sustained chaperone levels detected in the WT (**Figure 3, A-D**). The unprimed *bzip60* were similar to WT, with no detectable level of chaperons compared to control. Loss of *bZIP60* partially impairs the mechanisms that aid in the retention of

375 chaperons during priming-mediated heat stress recovery, which needs to be further investigated.
376 Taken together these results underscores the importance of bZIP60 for efficient priming-mediated
377 acquired thermotolerance in Arabidopsis.

378 Discussion

379 Priming is a phenomenon which renders the plants to mount a robust response to future stresses (biotic
380 or abiotic), thereby facilitate their growth and survival (Conrath, 2011; Wilkinson et al., 2019).
381 Previous studies identified the regulatory mechanisms underlying priming-mediated acquired
382 thermotolerance operating at the transcriptional and the epigenetic level (Crespo-Salvador et al., 2018;
383 Kappel et al., 2023; Lämke et al., 2016; Oberkofler et al., 2021). Majority of these studies focused on
384 understanding the priming-induced responses post few days of recovery before exposing to lethal heat
385 stress (Liu et al., 2021; Sedaghatmehr et al., 2021). Here, we aimed at understanding the short-term
386 priming-mediated acquired thermotolerance. Findings from our study demonstrate the impact of
387 priming on the regulation of protein homeostasis, thereby the ER status, to promote heat stress
388 tolerance. Heat stress has a profound impact on the thermolabile molecular complexes, resulting in
389 protein dysfunction and aggregation (McLoughlin et al., 2019; Mittler et al., 2012; Rosenzweig et al.,
390 2019), thereby promoting dysregulation of ER (Nakajima & Suzuki, 2013). Thus, it was imperative
391 for us to study if priming-mediated acquired thermotolerance involves regulation of proteostasis.
392 Thermopriming imparts significant tolerance to noxious heat stress, where the unprimed seedlings fail
393 to survive (**Figure 1, B-D**). We show that upon heat stress, dynamic regulation of autophagy and
394 chaperone activity evoked by priming, aids in the seedling survival. For instance, exposure to lethal
395 heat stress with or without priming triggers autophagy, however, during post stress recovery, it reverts
396 back to base levels in the primed alone (**Figure 2, C-F**). The reset of autophagy response observed in
397 the primed seedlings coincides with elevated chaperone abundance (**Figure 3, A-D**), suggesting
398 efficient maintenance of cellular homeostasis. Contrarily, in the absence of priming, heat-triggered
399 autophagy response remains longer with minimal to no induction of chaperones (**Figure 2, C-F;**
400 **Figure 3, A-D**), leading to an imbalance in the cellular protein homeostasis. Such disrupted
401 proteostasis may result in the accumulation of toxic byproducts, imposing heavy energy costs,
402 ultimately affecting growth and survival of the seedlings.

403 Several previous studies have demonstrated the regulation of autophagy through TOR/SnRK-
404 dependent pathways (Pu et al., 2017; Signorelli et al., 2019; Soto-Burgos & Bassham, 2017; Yang et

al., 2023). The TOR-Glucose signaling axis has also been identified to reprogram the transcriptome to promote heat stress tolerance in Arabidopsis (Sharma et al., 2021). However, our study suggests that TOR and SnRK are not involved in modulating the priming-induced autophagy response under heat stress (**Figure 4**). Notably, TOR-independent mechanisms modulate autophagy in response to oxidative and ER stress-inducing conditions (Pu et al., 2017). In order to test if priming modulates ER status to aid in heat stress tolerance, we monitored ER-GFP and IRE1-mediated *bZIP60* splicing as a marker for unfolded protein response (UPR) activation (Deng et al., 2011; Howell, 2013). Our findings suggest that exposure to priming temperature itself triggers ER stress and the associated UPR, which subsides during recovery post heat stress (**Figure 5A**). On the contrary, unprimed seedlings display sustained ER stress post heat trigger (**Figure 5A**), resulting in dysfunctional ER (**Figure 5B**), ultimately triggering cell death at later time points (**Figure 1B**). Further, to delineate the IRE1-RIDD mediated regulation of ER status upon heat stress, we checked the RIDD activity by analyzing the expression of its downstream target genes *viz. BGLU21*, *ROSY1* and *PR-14*. Interestingly, RIDD activity is reported to be anti-correlated with autophagy response (Bao et al., 2018). Although how RIDD target genes lead to the inhibition of autophagy are yet to be understood. Previous report suggests that BGLU21 (β-glucosidase 21), ROSY1 (interactor of synaptotagmin 1), and PR-14 (pathogenesis-related protein 14) proteins might interfere with the lipidation of ATG8, a crucial step for autophagosome formation, thereby affecting autophagy (Bao et al., 2018). Interestingly, we observed reduced level of RIDD target gene expression (autophagy inhibitors: *BGLU21*, *ROSY1*, *PR-14*) (**Figure 5, C-E**), which correlated with induced autophagy (**Figure 2, C-F**), both in primed and unprimed post heat stress. However, such response was restored quickly by one day post heat stress in the primed seedlings only, thereby aiding in their survival (**Figure 5, C-E**). In contrast, the induced and reduced autophagy and autophagy inhibitors, respectively, may lead to the disruption of proteostasis in the unprimed, ultimately leading to death of the seedlings. Furthermore, analysis of the *bzip60* knockout mutant suggests that bZIP60 is required for mounting efficient priming-induced heat stress response, as the mutant seedlings display reduced priming-mediated growth recovery (**Figure 6**) and HSP levels compared to WT (**Figure 7**). Future studies are required to gain insights into the role of bZIP60 in the regulation of priming-mediated acquired thermotolerance.

Taken together, our findings suggest that efficient maintenance of ER homeostasis through the dynamic regulation of autophagy, and chaperon levels evoked by priming, aids in seedling growth and

survival upon heat stress (**Figure 8; Supplementary Figure 7**). However, absence of priming leads to disrupted ER status in the unprimed samples, imposing heavy toll on ER, resulting in dysregulation of protein homeostasis. This leads to consequent loss of cellular proteome, thereby resulting in death of the seedlings. We believe that our current study for the first time underscores the close connection between priming stimulus and well-regulated dynamic changes associated with unfolded protein response in the ER compartment. We wonder if there are additional transcription factors compensating for the loss of bZIP60 that transduce the information between ER stress and chromatin thereby leading to subsequent epigenetic changes in the system.

Acknowledgements

MB acknowledges the Council of Scientific and Industrial Research (CSIR), Govt. of India for the fellowship. This work was supported by funding from the Department of Science and Technology (Sir JC Bose Fellowship – for BJR), Science and Engineering Research Board (SPG/2021/004000) and intramural support from IISER Tirupati (ADA and BJR), University of Hyderabad (BJR).

Authors Contribution

ADA and BJR conceptualized the research question and supervised the work. MB, ADA and BJR contributed to designing the experiments, interpretation of the results and preparation of the figures. MB performed the experiments. MB and ADA wrote the manuscript. ADA and BJR edited the manuscript. All authors read the manuscript.

Competing Interests

The authors have no competing interest

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Figure Legends

Figure 1: Priming improves survival under severe heat stress (A) The experimental paradigm is schematised where primed condition includes exposure to 37°C, a priming stimulus, followed by a brief recovery at optimal growth temperature of 22°C, then exposure to triggering heat stimulus of 45°C for indicated duration of time period. Direct exposure to triggering heat stress (45°C) represents the unprimed condition. Optimal growth temperature of 22°C, served as control. (B) Seedling growth was monitored in the primed, unprimed and control conditions as a function of time at 22°C during the post stress period. (C) The growth in Fig. 1B is quantified as number of leaves per seedling (No. of individual seedlings (n) =32) at 0, 1, 3 and 5 days post-stress. (D) Root length (n=5) was quantified at different time points post heat stress. Despite an initial growth halt observed, primed plants were observed to grow similar to that of control, while unprimed underwent a total growth arrest, and eventually failed to survive. *The data are presented as mean with error bars depicting standard deviation. Statistical significance for leaf number and root length measurements was calculated using two-way ANOVA (**** p <0.0001; ns – not significant) where the Asterix in figure (D) depicts the statistical comparison between primed and unprimed samples.*

Figure 2: Priming temporally regulates the autophagy under heat stress (A) The schematics of experimental paradigm is represented where AP (After Priming) and Day 0-3 denote timepoints where samples were collected. (B) SDS-PAGE analysis indicates that unprimed seedlings undergo total protein degradation by the end of 3 days post stress, while the primed resist the same. (C-D) Western blot analysis of day-wise samples (post stress) probing ATG8 and α-Tub revealed that primed seedlings show similar autophagy levels compared to control and primed-control (only exposed to 37°C). However, unprimed seedlings display surge in the ATG8 levels compared to control, suggesting induced

autophagy. (E) Schematics with samples collected at shorter intervals post-stress where BT indicates 'before triggering' timepoint. (F) Western blot analysis of hour-wise, post stress samples probing ATG8 and α-Tub revealed that primed seedlings exhibit a dwindling autophagy pattern wherein ATG8 levels peak by 0h and gradually drop to basal level by 6-8h of recovery, whereas the unprimed seedlings show persistent autophagy with the levels starting to peak at 2h that remain high much longer, beyond 8h (refer Fig. 2D).

Figure 3: Priming leads to increase in chaperone activity at both protein and transcript level (A-D) Western blot analysis of HSP17.6, HSP21, HSP70, HSP90, HSP101 and α-Tub proteins for various samples at the post heat stress timepoints reveals that priming leads to increase in the abundance of HSPs which persist even after the removal of stress. In contrast, unprimed samples do not show any increase in HSPs compared to control. (E-I) qPCR analysis of *HSP18.2*, *HSP21*, *HSP22*, *HSP70* and *HSP101* was performed for all the conditions at indicated time points. Expression changes reflect fold change compared to control. In the primed, *HSPs* get highly induced upon priming and triggering compared to control. In contrast, in the unprimed, induced expression for most of the *HSPs* is observed only by 1-day post heat stress. Interestingly, induced expression of *HSPs* is detected in the primed control at after triggering time point, which subsides with time. *The data are presented as mean with error bars depicting standard deviation. Statistical significance was calculated using one-way ANOVA (**** p <0.0001; *** p <0.001; ** p <0.01; * p <0.05; ns – not significant).*

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Figure 5: Primed seedlings recover from ER stress while the unprimed exhibit sustained ER stress (A) ER stress can be determined by the presence of *bZIP60* spliced form (*bZIP60s*). RT-PCR gel

analysis of *bZIP60s*, *bZIP60* and *ACT2* for various samples at given time points post heat stress indicates that ER in unprimed seedlings remains stressed, even after the removal of stress, while primed samples recover from ER stress. (B) Confocal images of ER-GFP displaying the endoplasmic reticulum (ER) in the leaves of seedlings across different conditions. Primed seedlings exhibit GFP signal similar to control, whereas the loss of GFP in unprimed suggests degrading ER. The panels are merge of brightfield and ER-GFP signal. (C-E) qPCR analysis of *BGLU21*, *PR-14* and *ROSY1* of various samples at the given post stress timepoints. Every condition is compared with its cognate control and represented as fold change. *The data are presented as mean with error bars depicting standard deviation. Statistical significance was calculated using one-way ANOVA (**** p <0.0001; *** p <0.001; ** p <0.01; * p <0.05; ns – not significant)*

Figure 6: bZIP60 deficiency leads to delayed recovery in growth, post-priming (A) Seedling growth of *bzip60* knockout mutant was monitored in the primed, unprimed and control as a function of days at 22°C post heat stress. (B-C) Root length (n=5) for wild-type and *bzip60* seedlings was quantified across days post heat stress with or without priming. Primed *bzip60* seedlings take longer time to grow as *bzip60* control post-stress, when compared to primed wild-type *vs.* wild-type control (refer Fig. 6B). (D) The phenotype of primed *vs.* control of mutant *vs.* wild-type 7-days post-stress was photographed. (E-F) Root to shoot length (n=6) analysis for Fig. 6D plants indicates a significant difference between *bzip60* primed *vs.* control samples 7 days post-stress. *The data are presented as mean with error bars depicting standard deviation. Statistical significance was calculated using student's t-test (** p <0.01; ns – not significant).*

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Figure 8: Schema representing priming-induced regulation of proteostasis aiding in mounting acquired thermotolerance.

Supplementary Figure Legends

Supplementary Figure 1: Autophagy regulatory network. The autophagy process is regulated by three major kinases – TOR, SnRK, and IRE1. TOR gets activated in energy-rich conditions and inhibits autophagy by phosphorylation of ATG1 and ATG13 proteins (Suttangkakul et al., 2011). SnRK gets activated under energy-depleted conditions and activates autophagy by inhibiting TOR complex formation (Soto-Burgos & Bassham, 2017). IRE1 is an ER membrane protein which activates upon ER stress. BIP protein dissociates from IRE1B following ER stress, and associates with unfolded proteins. Following detachment of BIP, IRE1B dimerizes leading to its activation. Upon IRE1B activation, bZIP60s protein is formed which enters the nucleus upregulating unfolded protein response (UPR) genes (Nawkar et al., 2018; Pastor-Cantizano et al., 2020). mRNA degradation based RIDD activity of IRE1B leads to degradation of autophagy negative regulators, promoting autophagy (Bao et al., 2018).

Supplementary Figure 2: TOR and SnRK activity do not change during priming (A-B) Quantification for TOR kinase activity by measuring pS6K to S6K ratios in various samples at given timepoints. (C-D) Quantification for SnRK activity by measuring pAMPK to α-Tub ratios across all the conditions at given timepoints. *The data are presented as mean with error bars depicting standard deviation. Statistical significance was calculated using one-way ANOVA (ns – not significant).*

Supplementary Figure 3: ER-GFP signal in leaf samples post-stress. Confocal image analysis of endoplasmic reticulum (ER-GFP) in the leaf samples across all the conditions at different time points post heat stress. All images are taken at 40x magnification.

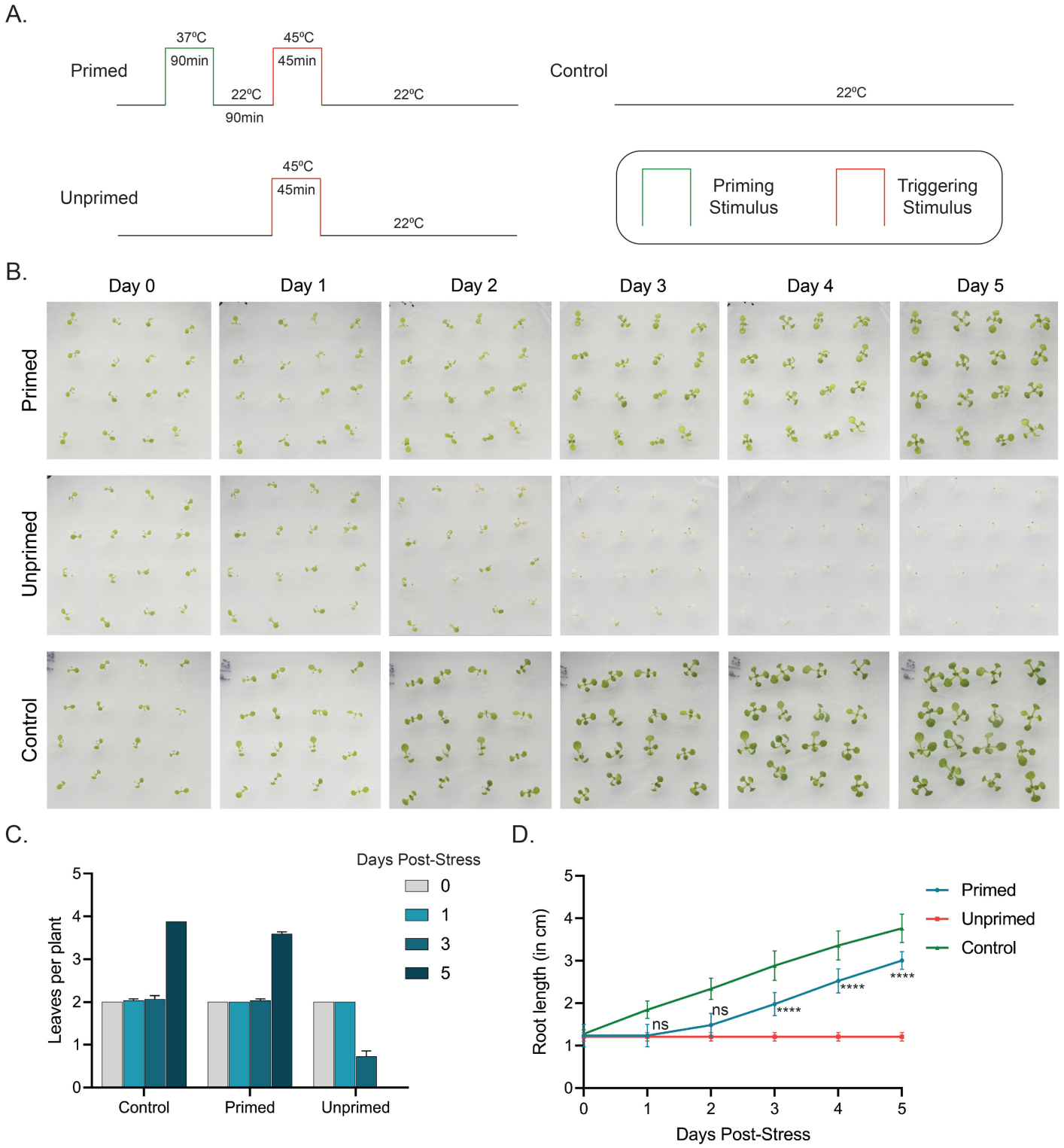
Supplementary Figure 4: Identification of homozygous knockout *bzip60* mutant lines by genotyping analysis. Genotyping by RT-PCR analysis for wild-type and mutant plants. Samples b1 to

b10 represent different *bzip60* mutant lines and NTC is no template control. Seeds harvested from samples b7, b8 and b10 identified as homozygous knockout lines were used for the experiments.

Supplementary Figure 5: Primed wild-type seedlings exhibit better survival than unprimed seedlings upon heat-stress. Seedling growth was monitored in the primed, unprimed and control samples as a function of time (days post stress) at optimal growth temperature of 22°C.

Supplementary Figure 6: *bzip60* mutants exhibit slower recovery in rosette growth than wild-type post priming. (A-B) Rosette area was quantified and compared during recovery post-priming in wild-type *vs.* mutant seedlings. *The data are presented as mean with error bars depicting standard deviation (n = 16). Statistical significance was calculated using two-way ANOVA (**** p <0.0001; *** p <0.001; * p <0.05; ns – not significant)*

Supplementary Figure 7: Model for IRE1 activation during priming (A) Proposed sequential changes during priming (B) Comparison of proposed sequential changes (at physiological and molecular levels) in wild-type *vs.* mutant *vs.* unprimed.



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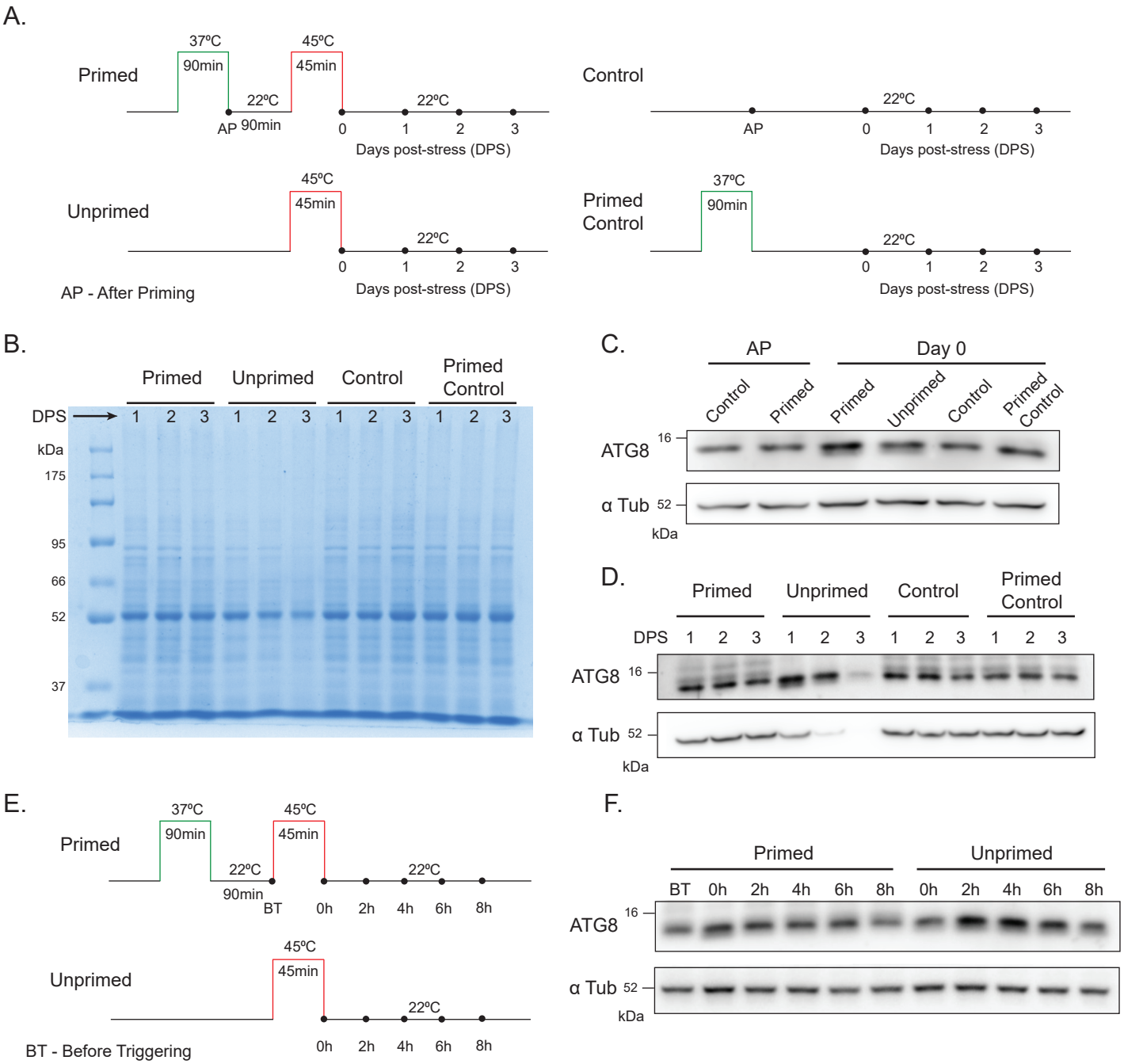

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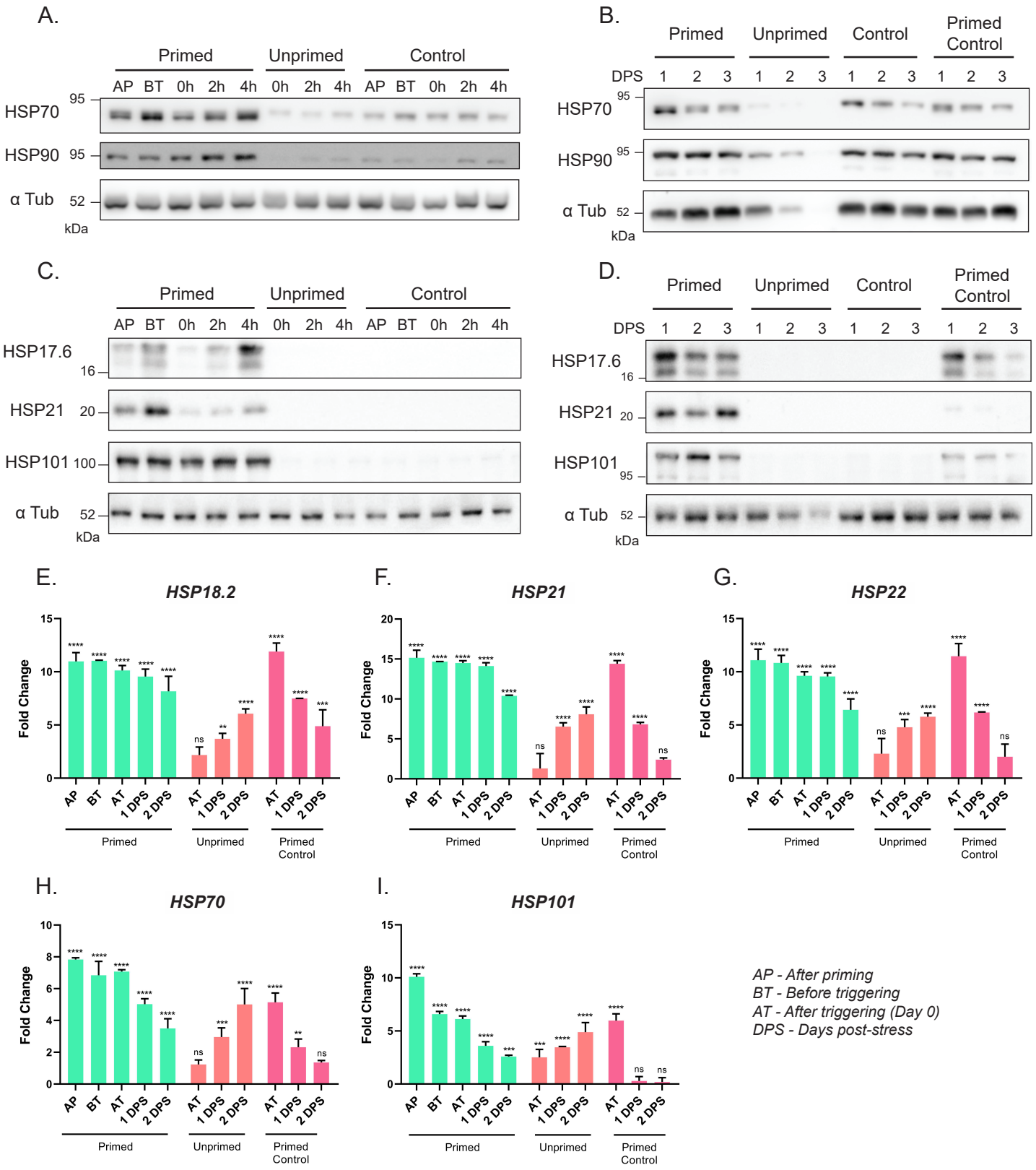

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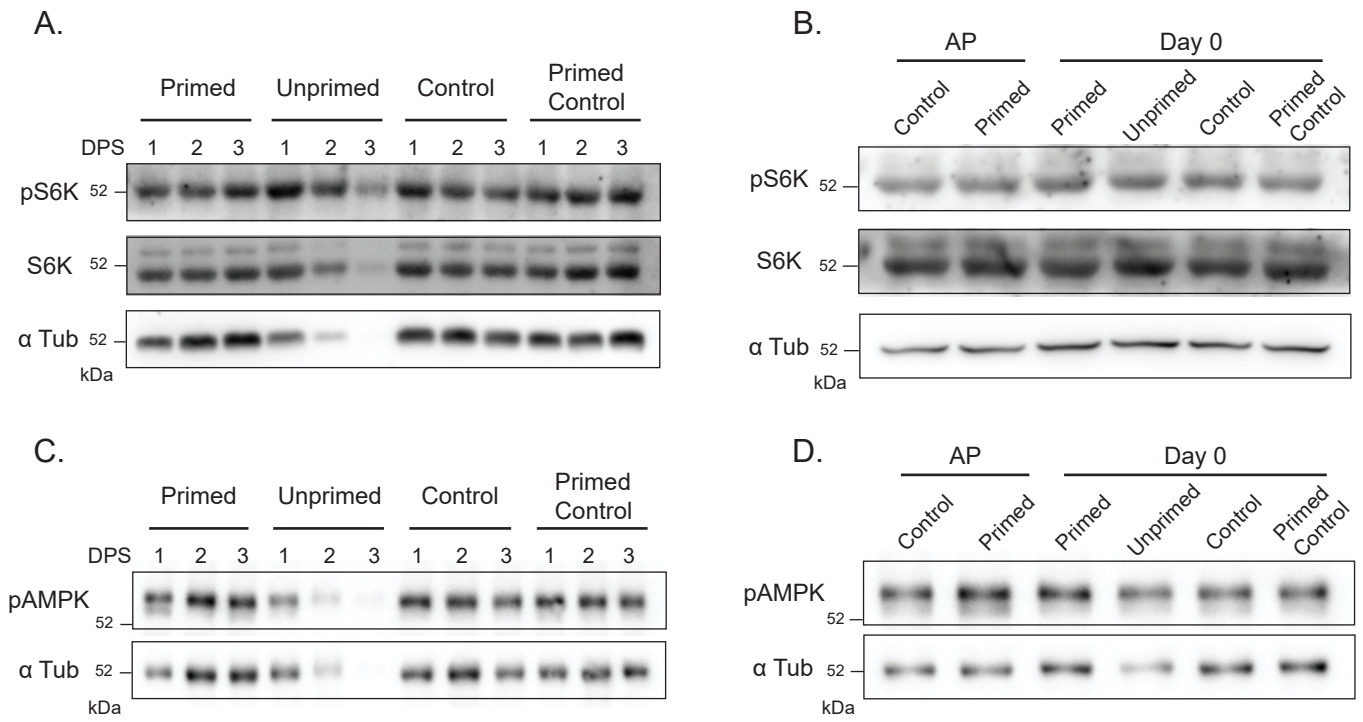

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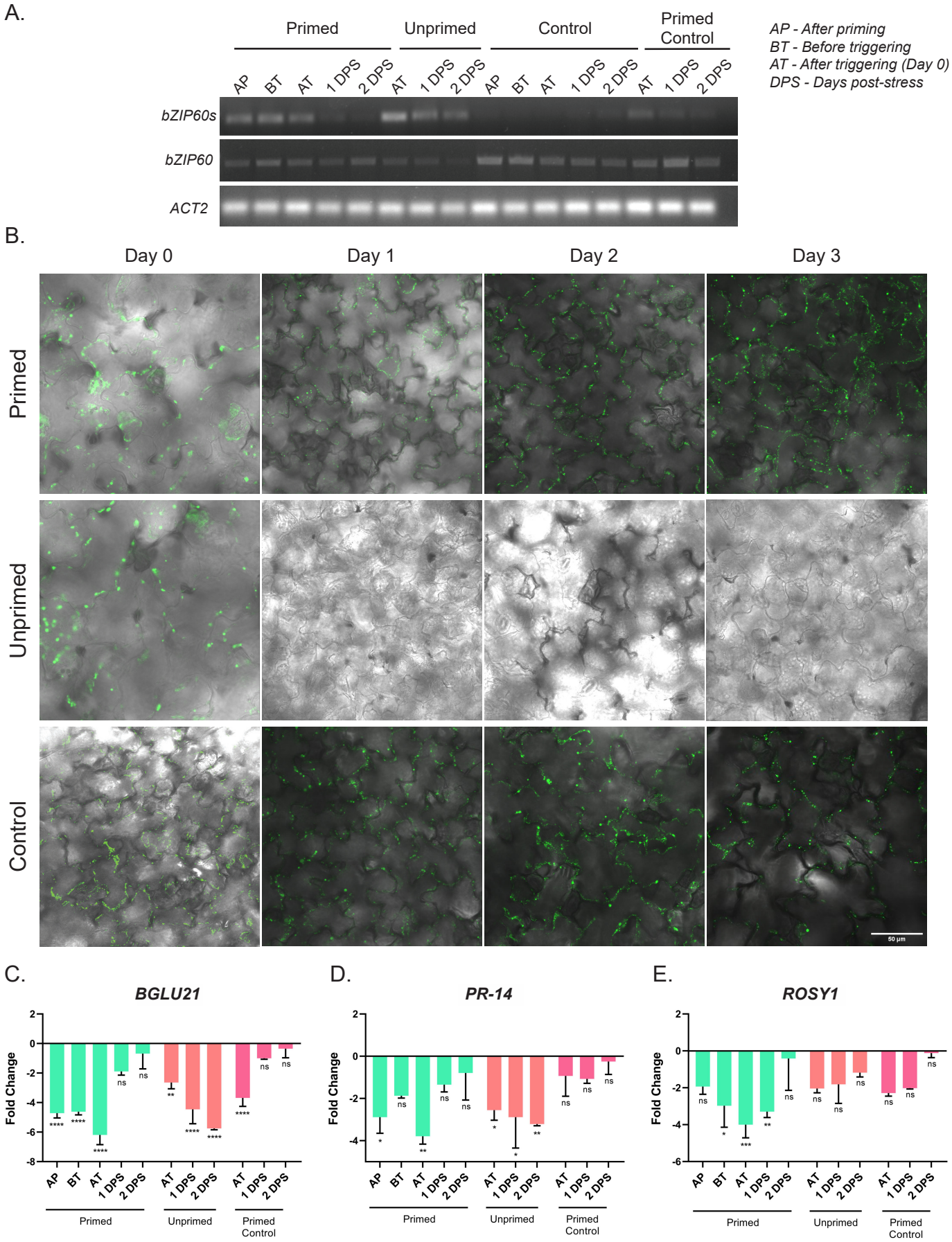

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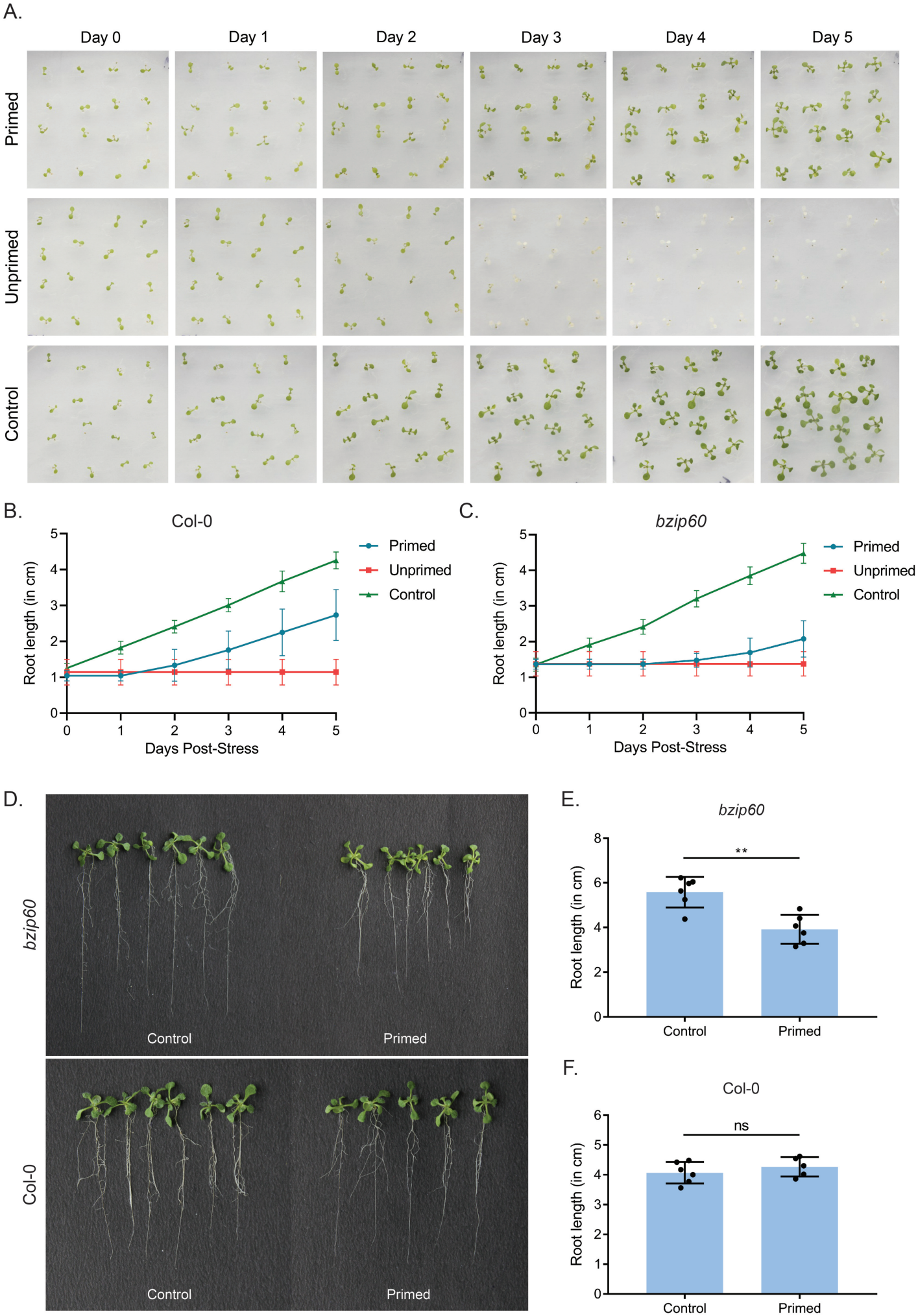

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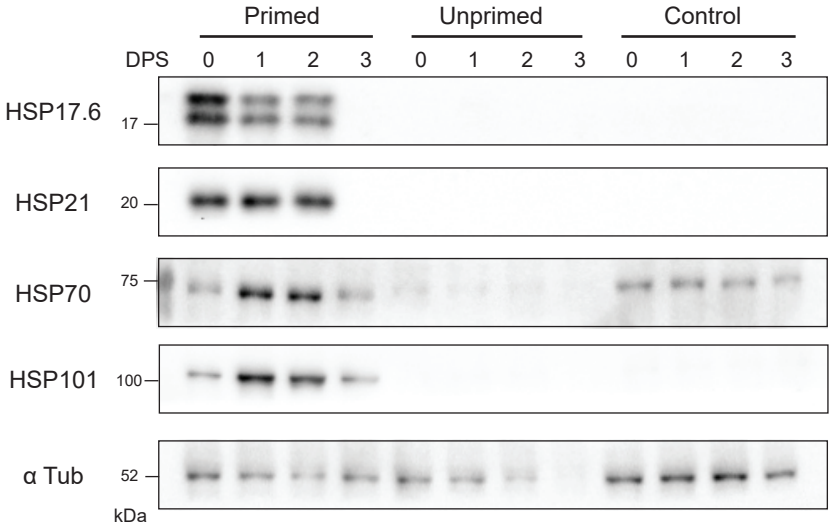

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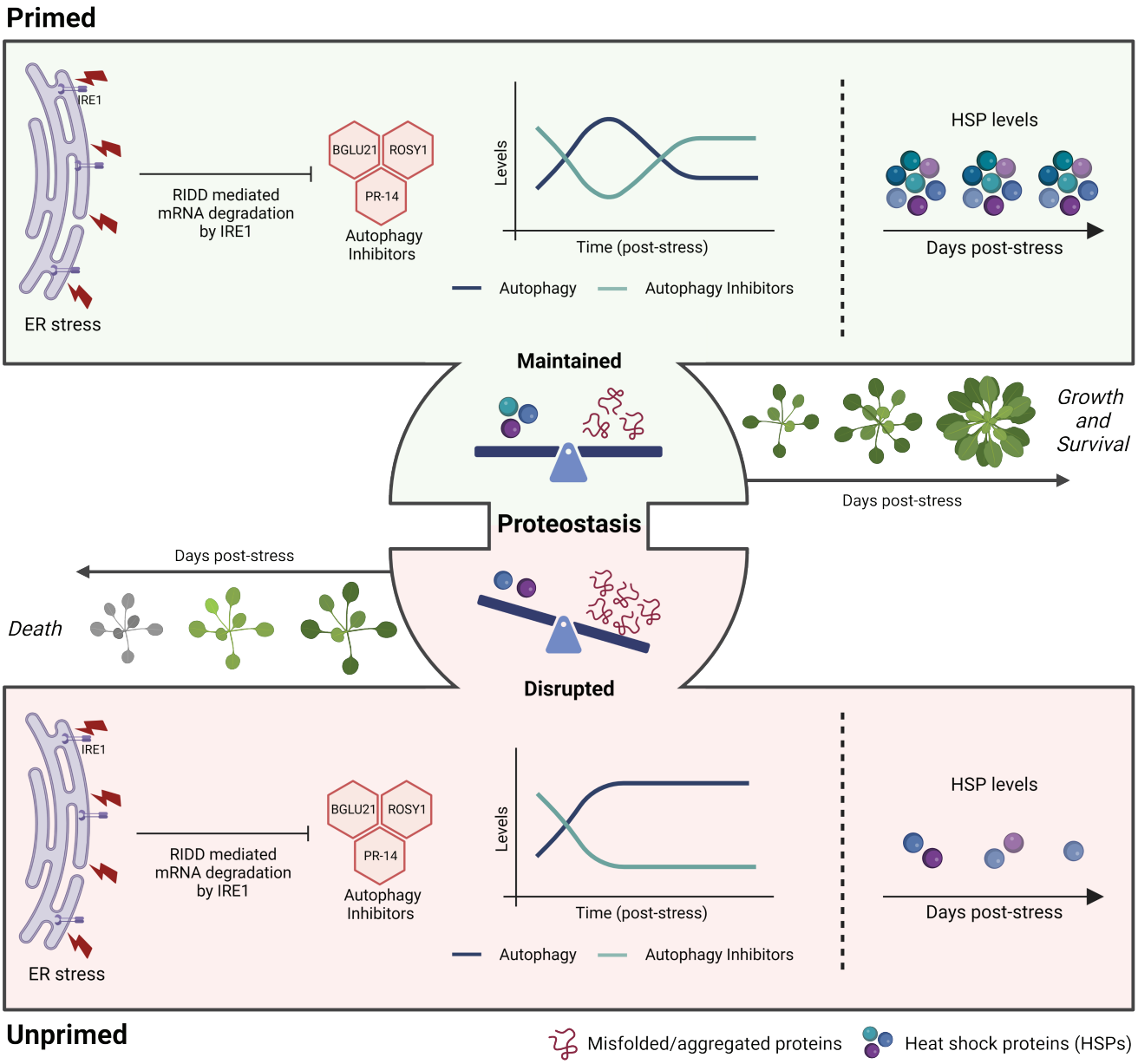

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