Proteomic plasticity undergirds stress responses in plants, and understanding such responses requires accurate measurement of the extent to which protein levels are adjusted to counter external stimuli. Here, we adapt bioorthogonal noncanonical amino acid tagging (BONCAT) to interrogate protein synthesis in vegetative Arabidopsis (Arabidopsis thaliana) seedlings. BONCAT relies on the translational incorporation of a noncanonical amino acid probe into cellular proteins. In this study, the probe is the Met surrogate azidohomoalanine (Aha), which carries a reactive azide moiety in its amino acid side chain. The azide handle in Aha can be selectively conjugated to dyes and functionalized beads to enable visualization and enrichment of newly synthesized proteins. We show that BONCAT is sensitive enough to detect Arabidopsis proteins synthesized within a 30-min interval defined by an Aha pulse and that the method can be used to detect proteins made under conditions of light stress, osmotic shock, salt stress, heat stress, and recovery from heat stress. We further establish that BONCAT can be coupled to tandem liquid chromatography-mass spectrometry to identify and quantify proteins synthesized during heat stress and recovery from heat stress. Our results are consistent with a model in which, upon the onset of heat stress, translation is rapidly reprogrammed to enhance the synthesis of stress mitigators and is again altered during recovery. All experiments were carried out with commercially available reagents, highlighting the accessibility of the BONCAT method to researchers interested in stress responses as well as translational and posttranslational regulation in plants.

Elevated temperatures, limited water resources, and high salt concentrations in arable soils are expected to profoundly and increasingly affect the productivity of crops in the coming years (Mickelbart et al., 2015).

Several promising marker-assisted breeding and genetic engineering strategies have been employed to help address this global challenge (Kasuga et al., 1999; Lopes and Reynolds, 2010; Mickelbart et al., 2015). For example, HVA1, a Late Embryogenesis Abundant gene from barley (Hordeum vulgare) shown to delay leaf wilting, was expressed in wheat (Triticum aestivum; Sivamani et al., 2000), rice (Oryza sativa; Xu et al., 1996), and corn (Zea mays; Nguyen and Sticklen, 2013) to produce salt-tolerant lines with higher water usage efficiency. Despite these early and promising successes, target selection remains a critical challenge associated with genetic engineering and marker-assisted breeding (Bita and Garets, 2013). Gaining further insight into the physiological mechanisms that govern stress tolerance and adaptation will improve our ability to rationally engineer crops.

Proteomic plasticity is a hallmark of the stress response in plants and was first shown over 35 years ago by incorporating radiolabeled amino acids into proteins synthesized during anaerobic stress in maize (Sachs et al., 1980) and heat stress in soybean (Glycine max; Key et al., 1981). With advances in mass spectrometry-based peptide identification, new strategies have been developed to measure the extent to which protein levels are adjusted in response to environmental stimuli (Huot et al., 1981). With advances in mass spectrometry-based peptide identification, new strategies have been developed to measure the extent to which protein levels are adjusted in response to environmental stimuli (Huot et al., 1981).
et al., 2014; Fristedt et al., 2015). But there are limitations to these technologies, which for the most part yield information about steady-state protein abundances. Moreover, traditional shotgun proteomics strategies provide reduced coverage in samples where a few highly abundant proteins predominate. For example, Rubisco comprises 30% to 60% of the leaf proteome and obstructs detection of less abundant proteins in leaf samples (Kim et al., 2013). To counter this problem specifically, Kim et al. (2013) developed a protamine sulfate precipitation method to selectively deplete Rubisco and thus enrich for less abundant proteins. Pulsed stable isotope labeling by amino acids in cell culture was introduced to monitor de novo protein synthesis, but poor label incorporation has prevented it from being widely adopted in plant systems. Other differential proteomics techniques, including stable isotope labeling by amino acids in cell culture (Lewandowska et al., 2013), hydroponic isotope labeling of entire plants (Bindschedler et al., 2008), $^{13}$CO$_2$ labeling (Chen et al., 2011), and isobaric tags for relative and absolute quantitation (Ge et al., 2013), have emerged in recent years as tools to probe in vivo protein synthesis. Notably, multiplexed quantification emerged in recent years as tools to probe in vivo protein and absolute quantitation (Ge et al., 2013), have labeling (Chen et al., 2011), and isobaric tags for relative and abundant proteins. Pulsed stable isotope labeling by amino acids in cell culture was introduced to monitor de novo protein synthesis, but poor label incorporation has prevented it from being widely adopted in plant systems. Other differential proteomics techniques, including stable isotope labeling by amino acids in cell culture (Lewandowska et al., 2013), hydroponic isotope labeling of entire plants (Bindschedler et al., 2008), $^{13}$CO$_2$ labeling (Chen et al., 2011), and isobaric tags for relative and absolute quantitation (Ge et al., 2013), have emerged in recent years as tools to probe in vivo protein synthesis.

In the BONCAT method, a noncanonical amino acid is pulsed into the cells of interest, where it is incorporated into newly synthesized proteins. Here we employed the Met surrogate azidohomoalanine (Aha), which carries an azide moiety that is amenable to biorthogonal click chemistry (Fig. 1). Bioorthogonal click chemistry refers to a set of reactions that are fast and highly selective in complex biological settings and that can be carried out under mild conditions (Sletten and Bertozzi, 2009; McKay and Finn, 2014). Pulse-labeling with Aha allows the investigator to exploit the selectivity of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) and the strain-promoted azide–alkyne cycloaddition (SPAAC) to visualize and enrich newly synthesized proteins (Fig. 1).

BONCAT has been used to study proteome-wide responses in mammalian and microbial culture (Dieterich et al., 2006; Zhang et al., 2014; Bagert et al., 2014) and to analyze subpopulations of cells in complex multicellular eukaryotes (Erdmann et al., 2015; Genheden et al., 2015; Yuet et al., 2015), but has yet to be employed in plant systems. Here, we applied BONCAT to Arabidopsis (Arabidopsis thaliana) to visualize proteins synthesized within 3 h after imposition of four significant abiotic perturbations: light stress (shift to darkness), high temperature, salt, and osmotic stress. We further coupled BONCAT-assisted enrichment to tandem liquid chromatography mass spectrometry (LC-MS/MS) to identify proteins synthesized de novo.

**Figure 1.** Scheme of BONCAT in native plant tissues. Aha is pulsed into aerial tissues where it can be incorporated into nascent proteins. The azide enables conjugation to fluorophores or beads for visualization or enrichment, respectively. CuAAC was used to conjugate TAMRA alkyne to nascent proteins. SPAAC (a biocompatible “click reaction”) was employed to conjugate nascent proteins to beads for enrichment.
Time-Resolved Analysis of Protein Synthesis

Aha Incorporation Is 2-Fold Higher at an Ambient Temperature of 37°C vs 22°C

While we observed protein labeling under each of the tested conditions (Fig. 3C), we note that labeling intensity was approximately 2-fold stronger (2.1 ± 0.4) under heat shock versus other tested conditions, suggesting that either translation rates are faster or Aha uptake is greater at higher temperatures. Several studies with cell suspension cultures and seedlings have shown decreases in protein synthesis rates at elevated temperatures (Barnett et al., 1979; Matsuura et al., 2010). We note here that although the ambient temperature in our experiment was 37°C, aerial tissues reached a peak temperature of only approximately 31°C (measured by infrared thermometer at the end of the stress period just prior to harvest), likely owing to evaporative cooling effects. Notably, Ishihara et al. (2015) report higher protein synthesis rates at 28°C than at 21°C in Arabidopsis.
seedlings using $^{13}$CO$_2$ labeling. These results are consistent with ours, where the tissues experience a temperature ramp from 22°C to a maximum of 31°C.

Separately, to rule out the possibility that increased incorporation of Aha was a result of greater uptake, we performed pulses with Aha concentrations up to 4 mM and labeled for 3 h at room temperature (Supplemental Fig. 1). Incorporation was measured by in-gel fluorescence. Under these conditions, Aha labeling appears to be saturated by 1 mM (Supplemental Fig. 1). These results demonstrate that increased incorporation at higher temperatures is likely not due to greater Aha uptake, but to increased protein synthesis.

**BONCAT Can Be Used to Enrich Newly Synthesized Proteins in Arabidopsis Seedlings**

We tested our ability to enrich newly synthesized proteins through a dibenzoazacyclooctyne (DBCO)-agarose bead pull-down method (Figs. 1 and 2A). Specifically, we pulsed Aha into Arabidopsis seedlings and allowed nascent proteins to be labeled for 3 h at room temperature. To remove nonprotein contaminants and obtain better quality samples for mass spectrometry, we isolated total protein from seedlings via trichloroacetic acid-acetone precipitation followed by phenol extraction (Wang et al., 2006; Wu et al., 2014). We resuspended samples in phosphate-buffered saline (PBS) supplemented with 1% SDS and checked for labeling by subjecting total protein to TAMRA-alkyne conjugation (Fig. 4B). A separate aliquot of total protein was subjected to SPAAC conditions to conjugate the Aha-labeled proteins to DBCO-agarose beads. The resin was washed extensively to remove nonspecifically bound proteins. Finally, Aha-labeled proteins were eluted from the resin via trypsin digestion and subjected to detergent removal and desalting prior to LC-MS/MS (Orbitap Elite) and analysis via MaxQuant software.

To quantify enrichment, we performed parallel pull downs from seedlings at 22°C labeled with Aha for 3 h and seedlings unexposed to Aha. We subjected each pull down to cleanup and LC-MS/MS and quantified the total sum of all Arabidopsis MS1 peptide extracted-ion chromatogram areas for both enriched and unlabelled mock samples. We found enrichments of at least 44-fold across three biological replicates.

**Identifying Candidate Proteins Involved in Thermotolerance and Recovery from Heat Stress**

After demonstrating the feasibility of labeling aerial tissues under stress conditions and developing an enrichment protocol, we sought to identify the de novo synthesized proteins that were involved in heat stress
and in recovery from heat stress by using a combination of BONCAT-assisted enrichment and LC-MS/MS. We opted to study heat stress because it has been well characterized in Arabidopsis at the level of translation (Matsuura et al., 2010, 2013; Mittler et al., 2012), and because thermotolerance has been shown to be induced by heating seedlings to 38°C for 90 min (Larkindale and Vierling, 2008), a timescale that is easily accessible to BONCAT analysis (Fig. 2B). Further, heat stress can be alleviated easily to monitor protein synthesis during recovery from stress (McLoughlin et al., 2016), an aspect of stress physiology that is relatively understudied at the level of protein synthesis.

Seedlings were treated with Aha and exposed to either heat shock (37°C) or room temperature control (22°C) conditions for 3 h. To study recovery, seedlings were first exposed to ambient heat shock conditions (37°C) for 3 h, allowed to recover for 4 h at room temperature (22°C), then treated with Aha at room temperature for 3 h. Experiments were carried out in biological triplicate for each condition (Fig. 4A).

Aha-labeled proteins in experimental and control samples were conjugated to DBCO-agarose beads and subjected to the enrichment protocol. In total, we identified and quantified 3,341 proteins across the four conditions [1 mM Aha control at room temperature (RT), 2 mM Aha control at RT, heat stress (37°C), and recovery following heat stress (37°C to 22°C; Supplemental Table S1)]. We identified 2,973 proteins across the room temperature control series 2 (2 mM Aha) and heat shock samples alone (Fig. 4C).

To assess enrichment of known heat-responsive proteins, we first filtered our dataset for proteins that were either significantly up-regulated in response to heat (P value < 0.01) or that were found in all three heat shock replicates and in none of the control series 2 (2 mM Aha) replicates [Supplemental Table S1; Label-Free Quantitation (LFQ) value = 0, Columns DV - DX)]. These criteria identified a total of 189 proteins as heat-responsive BONCAT-enriched proteins (Supplemental Table S1; Column EL). Proteins with a gene ontology (GO) annotation of “Response to Heat” were found to be significantly overrepresented (P value 8 x 10^{-22}; Fisher’s exact test) in the population of heat-responsive BONCAT-enriched proteins. These results clearly demonstrate that the BONCAT method detects enrichment of de novo synthesized “heat-responsive proteins” in response to 3 h of heat stress. We note that this assay detected proteins encoded by nuclear, mitochondrial, and plastid genes, showing that Aha is incorporated into proteins synthesized in different cellular compartments.

We constructed a volcano plot of proteins shared between heat shock samples and control series two samples to visualize proteins with statistically significant fold-changes (Fig. 4D). Our list of up-regulated proteins contains many known heat stress markers, including ClpB1, Hsp90-1, probable mediator of RNA polymerase II transcription subunit 37c, and Heat Shock Protein (HSP)70-5 (Queitsch et al., 2000; Lin et al., 2001; Sung et al., 2001; Takahashi et al., 2003; Yamada and Nishimura, 2008). Our analysis also identified proteins with statistically significant fold-changes that have not been annotated previously.

We performed principal component analysis on the basis of normalized LFQ values for each protein (Fig. 5). We found three distinct clusters: control samples, heat shock samples, and recovery samples. These results illustrate the repeatability of biological replicates in BONCAT analysis, and the clustering of the two control series (1 mM and 2 mM Aha) suggests that Aha does not cause significant perturbation of protein synthesis at
these concentrations. Furthermore, the separate clustering of the control and recovery samples shows that metabolism does not simply return to the preimposition state following heat stress.

Next, we constructed heat maps to compare protein levels across conditions (Fig. 6; Supplemental Table S1; Supplemental Fig. S2). This analysis demonstrates the distinction in BONCAT-identified proteins under the three conditions, including the marked up-regulation of heat response proteins under heat shock. Notably, many BONCAT-labeled proteins highly expressed during heat shock are synthesized at lower levels during the recovery period than under control conditions, clearly demonstrating that seedlings rapidly adjust to changing conditions in part by altering the synthesis of proteins.

To validate our BONCAT results, we performed immunoblot detection of two up-regulated proteins: ClpB1 (HSP101) and HSP 70-5. For this purpose, 5-d-old seedlings were grown identically to those in the BONCAT screen, then exposed to 22°C for 3 h (control), 37°C for 3 h (heat shock), or 37°C for 3 h then 22°C for 7 h (recovery; these conditions mimic the 4-h “rest” period plus the 3-h labeling period in the BONCAT experiment). We then extracted total protein in a procedure identical to protein extraction for analysis by LC-MS/MS. As anticipated, we observed strong induction for both ClpB1 and HSP 70-5 under heat stress (Fig. 7). Importantly, immunoblotting detected differences in abundance across treatment samples, irrespective of time of synthesis. In contrast, BONCAT measures protein synthesized within specified time frames.

Most of the proteins highly up-regulated during heat shock are down-regulated during the recovery period according to BONCAT detection (Fig. 6; Supplemental Fig. S2). We hypothesized that these highly up-regulated proteins would not be degraded rapidly during the recovery period because thermal priming, a thermotolerance mechanism, has been shown to occur under similar heat stress conditions on similar timescales (Larkindale and Vierling, 2008; Mittler et al., 2012). The results of our immunoblotting support the hypothesis. Specifically, ClpB1 and HSP70-5 were not degraded during the recovery from heat shock (Fig. 7), although their de novo synthesis was significantly reduced (Supplemental Table S1). Therefore, ClpB1 and HSP70-5 are stable over the course of our experiment.

BONCAT As a Hypothesis Generator

Gratifyingly, the BONCAT screen revealed 80 validated biomarkers (GO molecular function “response to heat”) of the heat stress response in Arabidopsis, including ClpB1 (HSP101) and HSP70-5. Notably, other proteins without an annotation of “response to heat” were also up-regulated during heat stress, suggesting a possible role in thermotolerance. Measuring proteins synthesized during recovery from heat stress yielded
new information. For example, during recovery we observed induction (2.75-fold) of zeaxanthin epoxidase, which catalyzes the first committed step in the biosynthesis of abscisic acid (Xiong and Zhu, 2003), a hormone known to promote stomatal closure (Morillon and Chispeels, 2001; Park et al., 2015). Stomatal closure, in turn, minimizes water loss due to evaporative cooling (Xiong and Zhu, 2003). Therefore, we hypothesize that under these conditions zeaxanthin epoxidase is down-regulated during heat stress to promote evaporative cooling, then up-regulated during recovery to promote stomatal closure and prevent desiccation. While detailed assignment of functional roles to heat-responsive proteins in the context of imposition and recovery is beyond the scope of this study, the observed changes in protein synthesis suggest mechanistic hypotheses that merit further evaluation.

DISCUSSION

Proteins are cellular workhorses that carry out tightly orchestrated developmental and adaptive programs. As sessile organisms, plants must retain a high degree of proteomic plasticity to enable rapid responses to a barrage of environmental cues (Huot et al., 2014; Fristedt et al., 2015; Mickelbart et al., 2015). In plants, as in most eukaryotes, mRNA abundance is often a poor proxy for protein levels (Ingolia, 2014; Vélez-Bermúdez and Schmidt, 2014; Fukao, 2015), as indicated by the discordance between total mRNA abundance and polyribosome-associated mRNAs under control and abiotic stress conditions (reviewed by Roy and von Arnim, 2013). To address this issue, we used BONCAT in intact Arabidopsis seedlings to identify and quantify proteins synthesized under conditions of abiotic stress. As implemented here, BONCAT enables identification of proteins synthesized over periods of a few hours and requires no manipulation of the translational machinery or presumption that a ribosome-associated mRNA will produce a stable protein (Fig. 2). Furthermore, endogenous Met levels do not need to be depleted for Aha to be incorporated into newly synthesized proteins.

In an initial test of the method, we found evidence that newly synthesized proteins can be labeled in seedlings subjected to a variety of abiotic stresses, including osmotic shock, high salt, and heat shock (Fig. 3). We then compared the populations of Arabidopsis proteins made under normal growth conditions to those made under conditions of mild heat stress and during recovery from heat stress (Fig. 4). Changes in protein synthesis in response to heat stress were readily observed in periods as short as 3 h. We used bioinformatic (Figs. 4–6) and immunoblotting analyses (Fig. 7; Supplemental Figs. S3 and S4) to validate the BONCAT-labeled proteins under three conditions. Unsurprisingly, the GO annotation “Response to Heat” was overrepresented among proteins up-regulated in response to heat shock. This result is congruent with previous studies showing selective induction of stress mitigators during mild heat stress. Our identification of de novo synthesized markers of the heat shock response provides validation of the BONCAT method as a tool for the study of proteome dynamics in plants. At the same time, we found many proteins, including presumably low abundance transcription factors like BIM1, that have not previously been associated with mitigation of heat stress, illustrating the potential value of time-resolved proteomic studies as a source of new mechanistic hypotheses.

Furthermore, we assessed protein synthesis during recovery from heat stress and found proteins most highly up-regulated in response to heat stress were synthesized during recovery at levels similar to or lower than pre-heat shock levels (Fig. 6). This result showcases a mechanism by which plants avoid synthesizing a surfeit of stress-associated proteins during rapidly changing conditions. Based on reduced de novo synthesis and maintained abundance, neither ClpB1 nor HSP 70-5 was rapidly degraded during the recovery period, consistent with previous work showing that plants can develop “thermal memory” (Larkindale and Vierling, 2008) that protects seedlings exposed to mild heat stress from subsequent assaults. This observation

Figure 7. Immunoblotting analysis of select proteins shown in BONCAT screen to be up-regulated in response to heat stress. A, ClpB1 (HSP101) and B, HSP70-5 were found to be highly up-regulated in response to heat stress. These proteins are not synthesized at high levels during the recovery period. Neither are they rapidly degraded during the recovery period. Steady-state ClpB1 levels during recovery are 0.95 ± 0.08 when the fluorescent signal of heat shock samples is normalized to 1.00. Relative fluorescence values are provided for the control (room temperature), heat shock, and recovery for HSP70-5. C, Loading control. All fluorescence signals were normalized to Colloidal Blue staining over the entire lane. Cont, control (room temperature), Rel. Fl, relative fluorescence; Rec, recovery.
suggests that the BONCAT method may also be applied to consider dynamics in protein turnover, as the protein detected will reflect both the synthesis and turnover within the time-period of labeling. Importantly, turnover and synthesis may be differentially regulated. Thus, the BONCAT method is unlikely to detect highly unstable proteins.

It is worth noting that our model employed vegetative seedlings. While understanding stress responses at all developmental stages is important to the engineering of more robust crops, previous studies have shown that yield losses are caused most prominently by assaults to reproductive tissues (Young et al., 2004; Zinn et al., 2010). The BONCAT method should prove useful across many developmental stages and tissues and under a wide variety of conditions, and can help unravel the genetic basis of traits involved in tolerance to both abiotic and biotic stresses. Notably, all reagents and probes used in this study are commercially available, further underscoring the ease by which this methodology can be readily adopted by laboratories with access to proteomic facilities.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (Arabidopsis thaliana) seeds (Col-0 accession) were subjected to vapor phase sterilization (100 mL of 6% v/v commercial bleach and 3 mL of 37% HCl v/v) for approximately 3 h. Seedlings were then dispensed on 40 mL solid media (100- x 15-mm petri dishes) containing 0.5 mM HCl (Sigma–Aldrich), 1X MS vitamins (Sigma–Aldrich), 0.3% (w/v) Suc, and 0.9% (w/v) Phytagel (Sigma–Aldrich). Plates were inverted at 4°C in darkness for 2 d to break seed dormancy. Plates were then placed at 22°C under 24-h daylight and positioned so that roots would grow into the medium. After germination, plants were allowed to grow for 5 d under constant light.

Seedling Flood Technique to Deliver Noncanonical Amino Acid

The protocol was adapted from the study of Ishiga et al. (2011). Aha labeling medium containing 0.5X MS salts (Sigma–Aldrich), 5% Suc, 0.0025% Silwet L-77 (Lehle Seeds), 1 mM Aha in 10 mM citrate buffer (pH 5.6) was freshly prepared prior to labeling the lysate by CuAAC for 2 min. After the medium was decanted, the petri dish labeling medium for 2 min. After the medium was decanted, the petri dish was replaced and the plates were wrapped in foil. The seedlings were exposed to short-term acute abiotic stresses for 3 h.

Enrichment Procedure and LC-MS/MS Sample Preparation

Samples were adjusted to a consistent volume (approximately 500 μL) with 1% SDS in PBS. Then, samples were diluted 2× with 8 M urea containing 1× EDTA-free protease inhibitor (Sigma–Aldrich, catalog no. 11873580001) and 0.85% NaCl.

Separately, DBCO-agarose beads (50 μL of 2× slurry; Click Chemistry Tools, catalog no. 1034-2) were washed 3× with 0.8% SDS in PBS. Then, protein samples were added to the resin and shaken at 1,200 rpm at room temperature overnight (for at least 16 h). The resin was washed with 1 M lithium acetate, then with 1 M ammonium acetate and stored at −20°C overnight to precipitate the protein. The tubes were then centrifuged at 16,000 RCF for 5 min at 4°C. The pellet was washed once with 100% methanol, then with 80% acetone. Next, the protein pellets were resuspended in PBS (pH 7.4, 1% SDS, 100 mM chloroacetamide, and 1 × Complete EDTA-free protease inhibitors (Sigma–Aldrich, catalog no. 11873580001) then pooled. Protein concentrations were measured via bicinchoninic acid assay. Approximately 0.3 mg total protein was carried through the enrichment procedure.

Cell Lysis and Protein Extraction for Enrichment

Samples were prepared in biological triplicate with one plate (approximately 0.5–0.7 g tissue fresh weight) representing one biological replicate. Samples were prepared with protocols adapted from Wang et al. (2006) and Wu et al. (2014). Frozen aerial tissues were macerated in liquid nitrogen with a mortar and pestle. The resultant powder (0.1–0.3 g) was transferred into a 1.7-mL tube (typically tissues from one plate were divided into six separate 1.7-mL tubes). The pellets were washed with 10% trichloroacetic acid in acetone by filling the tubes, mixing them well by vortexing then centrifuging at 16,000 RCF for 5 min at 4°C. The supernatant was removed. The pellet was washed with 80% methanol containing 0.1 M ammonium acetate then with 80% acetone. The pellets were allowed to dry at room temperature for 15 min to remove residual acetone. Next, 0.8 mL of liquefied phenol (pH 8.0, Sigma–Aldrich, catalog no. P4557) and 0.8 mL of SDS dense buffer (30% Suc, 2% SDS, 0.15% sodium azide (to prevent bacterial growth), 0.1% Triton X-100, pH 8.0, and 2-mercaptoethanol to a final concentration of 5% added fresh) were added to the tube. The contents were mixed thoroughly and incubated for 5 min at room temperature in a fume hood. Next the tubes were centrifuged at 16,000 RCF for 3 min at 4°C. The supernatant was transferred into a new 1.7-mL tube while taking precautions not to disturb the middle SDS interface. The 1.7-mL Eppendorf tube was filled with methanol containing 0.1 M ammonium acetate and stored at −20°C overnight to precipitate the protein. The tubes were then centrifuged at 16,000 RCF for 5 min at 4°C. The pellet was washed once with 100% methanol, then with 80% acetone. Next, the protein pellets were resuspended in PBS (pH 7.4, 1% SDS, 100 mM chloroacetamide, and 1× Complete EDTA-free protease inhibitors (Sigma–Aldrich, catalog no. 11873580001) then pooled. Protein concentrations were measured via bicinchoninic acid assay. Approximately 0.3 mg total protein was carried through the enrichment procedure.

In-Gel Fluorescence to Monitor Protein Labeling

After labeling with Aha, aerial tissues were immediately harvested, flash frozen with liquid nitrogen, and stored at −80°C until subsequent workup steps. Frozen tissues were macerated in liquid nitrogen. Frozen powder was immediately transferred into an Eppendorf tube containing 1 mL of lysis buffer (100 mM Tris, pH 8 containing 4% w/v SDS). Lysates were subjected to sonication at 80°C for 40 min, then subjected to further heating at 95°C for 30 min. Cellular debris was removed by centrifugation at 16,100 relative centrifugal force (RCF) RCF for 5 min. The conditions for labeling the lysate by CuAAC for in-gel fluorescence were based on a previously published protocol (Hong et al., 2009). Protein concentrations in the cleared lysate were measured via bicinchoninic acid assay. Forty micrograms of protein lysate was added to PBS to a final total volume of 221 μL. In a separate tube, the alkynyl dye, copper (II) sulfate, and tris-(hydroxypropyl)triazolylmethylamine were premixed and allowed to react for 3 min in the dark. Then, aminoguanidine HCl and sodium ascorbate were added to the lysate-PBS mixture. Final concentrations were as follows: alkynyl dye (2.5 μM), CuSO4 (1 μM), tris-(hydroxypropyl)triazolylmethylamine (5 μM), aminoguanidine HCl (5 μM), and sodium ascorbate (5 μM). All components were gently mixed (one inversion) and allowed to react for 15 min in the dark at room temperature without shaking. Proteins were then extracted by methanol/chloroform/water precipitation. Pellets were washed extensively (at least three times). The pellet was then dissolved in 2× SDS sample loading buffer and sonicated for 30 min at 80°C. Samples were heated to 95°C for 5 min and electrophoresed in precast 4% to 12% Bis-Tris polyacrylamide gels. The gel was washed with a fixing solution (50% water, 40% ethanol, 10% acetic acid) in the fume hood for 10 min and rinsed twice with water (2× 10 min) prior to being subjected to fluorescence imaging with an excitation laser at 532 nm and an emission band pass filter at 580 nm (Typhoon GE Healthcare). After fluorescence imaging, the gel was stained with Colloidal Blue (InstantBlue, Expeoned) for 1 h and imaged to ensure equal protein loading among all lanes. To obtain relative fluorescence values, signal intensity was measured for the entire lane in both the TAMRA (fluorescence) channel and the colloidal blue channel. Next, the ratio of fluorescence intensity to colloidal blue intensity was calculated and normalized to the control lane. Calculating the relative fluorescence values as ratios in this manner allows for comparison between lanes even if lane loading varies slightly.
8 × 5 mL 0.8% SDS in PBS, 8 × 5 mL 8 M urea, 8 × 5 mL 20% acetonitrile (ACN). For the second wash with each solution, the column was capped and allowed to sit for 10 min prior to draining. After the washes, beads were transferred to an Eppendorf tube with 10% ACN in 50 mM ammonium bicarbonate. Tubes were centrifuged for 5 min at 2,000 g and the liquid was removed. Then, 100 µL 10% ACN in 50 mM ammonium bicarbonate was added to the beads and 100 ng trypsin was added. The beads were shaken at 1,200 rpm and 37°C overnight. The supernatant was collected, and beads were washed twice with 150 µL 20% ACN. Supernatants from the 20% ACN washes were pooled with the supernatant from the overnight tryptic digest. Pooled supernatants were filtered to remove any beads that carried through, then dried on a speedvac.

Digested peptides were redissolved in 100 µL 50 mM ammonium bicarbonate and treated with HiPRR detergent removal resin (Thermo Fisher Scientific, catalog no. 88306). Finally, digested peptides were subjected to desalting clean-up step with a ZipTip (C18).

**LC-MS/MS Analysis**

Trypsin-digested samples were subjected to LC-MS/MS analysis on a nanoFlow LC system, EASY-nLC II, (Thermo Fisher Scientific) coupled to an Orbitrap Elite Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with a nanospray Flex ion source, essentially as described previously (Kalli et al., 2013).

Briefly, for the EASY-nLC II system, solvent A consisted of 97.8% H2O, 2% ACN, and 0.2% formic acid and solvent B consisted of 19.8% H2O, 80% ACN, and 0.2% formic acid. A 50-µL self-pack Picofrit column (New Objective, Inc.) with coated tip was packed in-house with 3.0 µm ReproSil-Pur C18AQ resin (120-Å pore size, Dr. Maisch) to 16 cm. Samples were directly loaded onto the column, and the column was washed twice with 150 µL 20% ACN, with a 120-min gradient at a flow rate of 320 nL/min. The gradient was as follows: 2% to 30% Solvent B (150 min), 30% to 100% B (1 min), and 100% B with a 120-min gradient at a flow rate of 3200 nL/min. The gradient was as follows: 2% to 30% Solvent B (150 min), 30% to 100% B (1 min), and 100% B (9 min). Eluted peptides were then ionized using a standard coated silica tip (New Objective) as an electrospray emitter and introduced into the mass spectrometer. The Orbitrap Elite was operated in a data-dependent mode, automatically alternating between a full-scan (m/z 400-1,600) in the Orbitrap and subsequent MS/MS scans of the 20 most abundant peaks in the linear ion trap (Top20 method). Data acquisition was controlled by Xcalibur 2.2 and Tune 2.7 software (Thermo Fisher Scientific). Raw mass spectrometry files have been uploaded to the Japan Proteome standard Repository (jPOSTrepo), a publicly available data repository (ProteomeXchange ID: PXD005577).

**Bioinformatic Analysis**

Raw files were processed and searched using MaxQuant (v. 1.5.3.30; Cox and Mann, 2008; Cox et al. 2011). Spectra were searched against the UniProt Arabidopsis database (33,333 sequences) and a contaminant database (245 sequences). A decoy database was generated by MaxQuant using a reversed protein sequence strategy to estimate the false discovery rate. Trypsin was specified as the digestion enzyme with up to two missed cleavages allowed. Oxidation of Met (+15.9949), protein N-terminal acetylation (+42.0106), and replacement of Met with Ala (−4.9863) were allowed as variable modifications. Carbamidomethylation of Cys (+57.0215) was specified as a fixed modification. Precursor ion tolerance was less than 4.5 ppm after recalibration and fragment ion tolerance was 0.5 D. "Match Between Runs" and LFQ were enabled in MaxQuant. Protein and peptide identifications had an estimated false discovery rate <1%. Significance of fold changes was calculated using the R package limma (Benjamini and Hochberg 1995; Smyth GK 2004; Ritchie et al., 2015). Heatmaps were created using GENE-E where the sample clustering was performed using the average linkage and Euclidean distance and the gene clustering was performed using the average linkage and 1-Pearson correlation coefficient. For heatmap visualization, proteins had to be quantified in at least two control samples and two "treated" samples (either heat shock or recovery). Relative protein expression was normalized individually for each protein so that the average control expression was zero.

**Immunoblotting**

Unconjugated primary antibodies against ClpB1 and HSP70-5 were purchased from Abcam. Goat antimouse IgG (H+L) (A-21235) and goat antirabbit IgG (H+L) (A-21429) secondary antibodies with conjugated Alexa Fluor were purchased from Life Technologies (Thermo Fisher Scientific). Lysate (10 µg) was electrophoresed in 4-12% precast Bis-Tris polyacrylamide gels for all western blots. The 0.2-µm nitrocellulose membranes were blocked with 3% w/v nonfat dry milk in TBST for 1 h prior to incubating with antibodies in TBST. Primary antibodies [anti-ClpB1 (Ab80121, 1:5,000) and anti-HSP70-5 (ab5439, 1:5,000)] were incubated with their respective membranes at 4°C overnight with 3% w/v nonfat dry milk prior to staining with the secondary antibody (1:5,000) for 1 h and imaging (PMT 400 V; 50 µm pixel size). Fluorescence signals were normalized to total signal from Collodial Blue staining for each sample. To ensure that samples were measured in the linear range for quantitation, 1.5 µg, 10 µg, and 15 µg total protein were loaded for each heat shock replicate, and fluorescence was measured after incubation with the anti-HSP70-5 antibody and the secondary antibody as described above (Supplemental Fig, S4A). Fluorescence intensity was plotted against total protein concentration, which shows that signals were in the linear range for accurate quantitation (r² = 0.994; Supplemental Fig, S4B).

**Accession Numbers**

Raw mass spectrometry files have been uploaded to the Japan Proteome standard Repository under accession number PXD005577.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Table S1.** Protein groups.

**Supplemental Figure S1.** Concentration series.

**Supplemental Figure S2A.** Heat map – all.

**Supplemental Figure S2B.** Heat map – ANOVA.

**Supplemental Figure S2C.** Heat map – GO: response to heat.

**Supplemental Figure S2D.** Heat map – Proteins regulated by heat stress.

**Supplemental Figure S2E.** Heat map – GO: stress response.

**Supplemental Figure S3.** Full image of ClpB1 (HSP101) and HSP70-5 western blots.

**Supplemental Figure S4.** HSP70-5 fluorescence measurements lie in linear range.

**ACKNOWLEDGMENTS**

We thank the laboratory of Professor Elliot Meyerowitz, especially Arnavaz Garda and Dr. Paul Tarr, for seeds, space in growth chambers, and helpful discussions. We also thank Roxana Eggleston-Rangel for helpful discussions on sample preparation for mass spectrometry.

Received November 17, 2016; accepted January 14, 2017; published January 19, 2017.

**LITERATURE CITED**


with 13C-carbon dioxide – an in vivo labeling system for pro teaseomics and metabolomics research. Proteome Sci 9: 9


