Bioorthogonal Non-Canonical Amino Acid Tagging (BONCAT) enables time-resolved analysis of protein synthesis in native plant tissue.

Weslee S. Glenn, Shannon E. Stone, Samuel H. Ho, Michael J. Sweredoski, Annie Moradian, Sonja Hess, Julia Bailey-Serres and David A. Tirrell

1 – Division of Chemistry and Chemical Engineering; California Institute of Technology; Pasadena, CA 91125
2 – Proteome Exploration Laboratory; California Institute of Technology; Pasadena, CA 91125
3 – Center for Plant Cell Biology; University of California at Riverside; Riverside, CA 92521

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Summary: Pulsing the non-canonical amino acid azidohomoalanine into Arabidopsis thaliana seedlings enables in-gel visualization, physical enrichment, and identification of newly synthesized proteins.

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Corresponding Author:
David A. Tirrell
California Institute of Technology
1200 E. California Blvd
MC 210-41
Pasadena, CA 91125
Telephone: (626) 395-3140
E-mail: tirrell@caltech.edu

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Abstract

Proteomic plasticity undergirds stress responses in plants, and understanding such responses requires accurate measurement of the extent to which proteins levels are adjusted to counter external stimuli. Here, we adapt bioorthogonal non-canonical amino acid tagging (BONCAT) to interrogate protein synthesis in vegetative Arabidopsis thaliana seedlings. BONCAT relies on the translational incorporation of a non-canonical amino acid (ncAA) probe into cellular proteins. In this study, the probe is the methionine 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 (LC-MS) 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 post-translational regulation in plants.

Introduction

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 (LEA) gene from barley (Hordeum vulgare L.) shown to delay leaf wilting, was expressed in wheat (Triticum aestivum L.; Sivimani et al., 2000), rice (Oryza sativa L.; Xu et al., 1996) and corn (Zea mays L.;
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 (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., 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 30–60% 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. 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 (pSILAC) 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 SILAC (Lewandowska et al., 2013), hydroponic isotope labeling of entire plants (HILEP; Bindschedler et al. 2008), $^{13}$CO$_2$ labeling (Chen et al. 2011) and isobaric tags for relative and absolute quantitation (iTRAQ; Ge et al., 2013, Pei et al. 2013), have emerged in recent years as tools to probe in vivo protein synthesis in plants. Notably, multiplexed quantifications with techniques such as iTRAQ are inherently problematic due to known interference problems when using ion trap sources (McAlister et al., 2014). Modern instruments with MultiNotch MS3 capabilities can overcome these limitations, but impose significant sensitivity penalties (McAlister et al., 2014). The instrumentation required for such sophisticated experiments is expensive and limits widespread use.
Identifying proteins that are translated within brief time intervals remains challenging because such proteins constitute a small fraction of the total proteome. While transcriptomic approaches offer good time resolution (on the minute time scale) (Kreps et al., 2002; Preston et al., 2009), transcript abundances and protein levels are frequently discordant (Vélez-Bermúdez and Schmidt, 2014; Fukao, 2015). Translating ribosome affinity purification (TRAP) (Zanetti et al., 2005; Mustroph et al., 2009; Jiao and Meyerowitz, 2010), followed by mRNA quantitation or ribosome footprint mapping (Juntawong et al., 2014), provides proxies for protein synthesis at specific time points in environmental or developmental processes. However, measurements of mRNA association with ribosomes and footprinting techniques may still fail to provide accurate information on changes in the proteome because of artifacts arising from harvesting and lysis steps (Ingolia, 2014) and turnover following protein synthesis. To complement the existing chemical biology toolkit for plant proteomics, we introduce bioorthogonal non-canonical amino acid tagging (BONCAT), which enables sensitive detection and identification of proteins synthesized within defined time intervals.

In the BONCAT method, a non-canonical amino acid is pulsed into the cells of interest, where it is incorporated into newly synthesized proteins. Here we employed the methionine surrogate azidohomoalanine (Aha), which carries an azide moiety that is amenable to bioorthogonal click chemistry (Figure 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 (Figure 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., 2015), and to analyze sub-populations 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 A. thaliana to visualize proteins synthesized within three hours 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 LC-MS to identify proteins synthesized \textit{de novo} during transient heat stress and a recovery period. The results from our screen are consistent with a model in which cellular energy resources are rapidly channeled to optimize a protective response at the onset of stress. We suggest that BONCAT should be broadly useful in the investigation of plant physiology and developmental plasticity.
Results

BONCAT can be used to label newly synthesized proteins in *A. thaliana* seedlings

We pulsed Aha (1 mM) into 5-day-old seedlings (see methods) and allowed labeling to proceed for up to 3 h (Figure 2). To determine whether Aha is taken up and incorporated into newly synthesized proteins, we first macerated aerial tissues of Aha-exposed seedlings grown under non-stress conditions with liquid nitrogen and then resuspended the resulting powder in a simple Tris buffer containing SDS. Total protein was isolated using chloroform-methanol precipitation, resuspended in a Tris buffer and treated with TAMRA-alkyne (Figure 2) under CuAAC conditions to conjugate the fluorophore to Aha-labeled proteins (Figure 1). We then resolved the proteins via one-dimensional SDS-PAGE and measured in-gel fluorescence (Figure 2B). Only Aha-labeled proteins are tagged with the fluorophore (Figure 2B and Figure 2C). We observed light labeling in aerial tissues in periods as short as 30 min (Figure 2B). These results confirm that Aha was both transported into intact tissues and activated by the endogenous methionyl-tRNA synthetase (MetRS).

To test the applicability of the BONCAT method to the study of stress responses, we used the Aha-labeling protocol described above, but also exposed the seedlings to a short-term acute abiotic stresses for 3 h, including a shift to darkness, osmotic shock (200 mM mannitol, a proxy for drought), high salt (300 mM NaCl) and heat shock (37 °C) (Figure 3). Only the salt stress caused a change in phenotype, where leaves collapsed slightly upon exposure (Figure 3B). Importantly, the seedlings exhibited no detectable difference in phenotype resulting from Aha exposure over the course of the experiment (Figure 3). We observed significant Aha labeling under all conditions in comparison with negative controls, where seedlings were grown under normal conditions but no Aha was added (Figure 3C). Equal amounts of total protein were loaded into each lane (Figure 3D) to allow labeling levels to be compared between conditions (Figure 3C).

Aha incorporation is two-fold higher at an ambient temperature of 37 °C vs 22 °C

While we observed protein labeling under each of the tested conditions (Figure 3C), we note that labeling intensity was approximately two-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 and Matsuura *et al.*, 2010). We note here that although the ambient temperature in our experiment was 37 °C, aerial tissues only reached a peak temperature of 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 13CO2 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 (SI Figure 1). Incorporation was measured by in-gel fluorescence. Under these conditions, Aha labeling appears to be saturated by 1 mM (SI Figure 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 *A. thaliana* seedlings**

We tested our ability to enrich newly synthesized proteins through a dibenzoxacyclooctyne (DBCO)-agarose bead pull-down method (Figure 1 and Figure 2A). Specifically, we pulsed Aha into *A. thaliana* seedlings and allowed nascent proteins to be labeled for 3 h at room temperature. To remove non-protein contaminants and obtain better quality samples for mass spectrometry, we isolated total protein from seedlings via trichloroacetic acid (TCA)-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 (Figure 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 non-specifically bound proteins. Finally, Aha-labeled proteins were eluted from the resin via trypsin digestion and subjected to detergent removal and desalting prior to tandem mass spectrometry (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 tandem LC-MS and quantified the total sum of all *A. thaliana* MS1 peptide extracted-ion chromatogram (XIC) areas for both enriched and unlabeled 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 *A. thaliana* at the level of translation (Matsuura *et al.*, 2010; Matsuura *et al.* 2012; 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 (Figure 2B). Further, heat stress can be alleviated easily to monitor protein synthesis during the 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 (Figure 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 3341 proteins across the four conditions [1 mM Aha control at 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 2973 proteins across the room temperature control series 2 (2 mM Aha) and heat shock samples alone (Figure 4C).

To assess enrichment of known heat-responsive proteins, we first filtered our dataset for proteins that were either significantly upregulated 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 [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 (Table S1; Column EL). Proteins with a gene ontogeny (GO) annotation of ‘Response to Heat’ were found to be significantly over-represented (p-value 8×10⁻²²; 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
task series 2 samples to visualize proteins with statistically significant fold-changes
(Figure 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 HSP70-5 (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 (Figure 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 pre-imposition
state following heat stress.

Next, we constructed heat maps to compare protein levels across conditions
(Figure 6; Supplemental Table S1; Supplemental Figure S2). This analysis demonstrates
the distinction in BONCAT-labeled proteins under the three conditions, including the
marked upregulation 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
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 (Figure 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 upregulated during heat shock are downregulated during the recovery period according to BONCAT detection (Figure 6; Figure S2). We hypothesized that these highly upregulated 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 Vierliing, 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 (Figure 7), although their de novo synthesis was significantly reduced (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 *A. thaliana*, including ClpB1 (HSP101) and HSP70-5. Notably, other proteins without an annotation of ‘response to heat’ were also upregulated during heat stress, suggesting a possible role in thermostolerance. Measuring proteins synthesized during recovery from heat stress yielded new information. For example, during recovery we observed induction (2.75-fold) of zeaxanthin epoxidase (ZEP), which catalyzes the first committed step in the biosynthesis of abscisic acid (Xiong and Zhu, 2003), a hormone known to promote stomatal closure (Morillo and Chrispeels, 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 ZEP is downregulated during heat stress to promote evaporative cooling, then upregulated 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.

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. Heat Shock Protein 70-5 (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. Abbreviations: Rel. Fl – relative fluorescence, Cont – control (room temperature), HS – Heat Shock, Rec – Recovery.
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; Velez-Bermudez 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 *A. thaliana* 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 (Figure 2). Furthermore, endogenous methionine 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 (Figure 3). We then compared the populations of *A. thaliana* proteins made under normal growth conditions to those made under conditions of mild heat stress and during recovery from heat stress (Figure 4). Changes in protein synthesis in response to heat stress were readily observed in periods as short as 3 h. We used bioinformatic (Figure 4; Figure 5 and Figure 6) and immunoblotting analyses (Figure 7; Figure S3 and Figure S4) to validate the BONCAT-labeled proteins under three conditions. Unsurprisingly, the GO annotation ‘Response to Heat’ was over-represented among proteins upregulated 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 upregulated in response to heat stress were synthesized during recovery at levels similar to or lower than pre-heat shock levels (Figure 6). This result showcases a mechanism by which plants avoid synthesizing a surfeit of stress-associated proteins during rapidly changing conditions. Based on reduced \textit{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 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 \textit{et al}., 2004; Zinn \textit{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.
Methods

Plant Growth Conditions

*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 x15 mm petri dishes) containing 0.5x strength Murishige Skoog (MS) salts (Sigma-Aldrich; St. Louis, MO), 1x MS vitamins (Sigma-Aldrich; St. Louis, MO), 0.3% (w/v) sucrose and 0.9% (w/v) Phytagel (Sigma-Aldrich; St. Louis, MO). 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 non-canonical amino acid

The protocol was adapted from the study of Ishiga *et al.* (2011). Aha labeling medium containing 0.5x Murishige Skoog (MS) salts (Sigma-Aldrich; St. Louis, MO), 5% sucrose, 0.0025% Silwet L-77 (Lehle Seeds, Round Rock, TX), 1 mM azidohomoalanine (Aha, Iris Biotech Gmbh; Marktredwitz, Germany) in 10 mM citrate buffer (pH 5.6) was freshly prepared prior to running the experiment. Seedlings were fully submerged in the Aha labeling medium for 2 min. After the medium was decanted, the petri dish covers were replaced and the plates were wrapped in foil. The seedlings were then exposed to short-term acute abiotic stresses for 3 h. Shift to darkness was accomplished by covering plates with foil. To approximate osmotic shock, Aha labeling medium was supplemented with 200 mM mannitol and pulsed onto seedlings during delivery of Aha. High salt stress was accomplished by supplementing Aha labeling medium with 300 mM NaCl. To heat shock samples, plates were placed in an oven at an ambient temperature of 37 °C immediately following the 2-min submersion in Aha labeling medium. All proteomics experiments were initiated on the morning of day 5 (germination is defined as day 0).

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 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 (BCA) assay. Forty micrograms of protein lysate was added to phosphate-buffered saline to a final total volume of 221 μL. In a separate tube, the alkynyl dye, copper (II) sulfate and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) 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 mM), THPTA (5 mM), aminoguanidine HCl (5 mM), sodium ascorbate (5 mM). 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 2X SDS sample loading buffer and sonicated for 30 min at 80 °C. Samples were heated to 95 °C for 5 min and electrophoresed in pre-cast 4-12% Bis-Tris polyacrylamide gels. The gel was washed with a fixing solution (50% water, 40% ethanol, 10% acetic acid) in the dark 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 imagining, the gel was stained with Colloidal Blue (InstantBlue, Expeadeon, San Diego, CA) 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.
Cell lysis and protein extraction for enrichment

Samples were prepared in biological triplicate with 1 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 (0.1-0.3g) was transferred into a 1.7-mL tube (typically tissues from 1 plate were divided into 6 separate 1.7-mL tubes). The pellets were washed with 10% trichloroacetic acid (TCA) in acetone by filling the tubes, mixing them well by vortexing then centrifuging at 16,000 RCF for 3 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 number P4557) and 0.8 mL of SDS dense buffer [30% sucrose, 2% SDS, 0.15% sodium azide (to prevent bacterial growth), 0.1 M Tris-HCl, 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 upper phenol phase was transferred into a new 1.7-mL tube while taking precautions not to disturb the middle SDS interface. The new 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 phosphate-buffered saline [PBS, pH 7.4, 1% SDS, 100 mM chloroacetamide and 1X cOmplete EDTA-free protease inhibitors (Sigma Aldrich, Catalog Number 11873580001)] then pooled. Protein concentrations were measured via BCA assay. Approximately 0.3 mg total protein was carried through the enrichment procedure.

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 2X with 8 M urea containing 1X EDTA-free protease inhibitor (Sigma Aldrich, Catalog Number 11873580001) and 0.85 M NaCl.
Separately, DBCO-agarose beads (50 uL of 2X slurry; Click Chemistry Tools, Catalog Number 1034-2) were washed 3X with 0.8% SDS in PBS. Then, protein samples were added to the resin and shaken at 1200 rpm at room temperature overnight (for at least 16 h). The resin was washed with 1 mL water. Then, 0.5 mL dithiothreitol (1 mM in 0.8% SDS in PBS) was added to the resin and incubated with shaking (1200 rpm) for 15 min at 70°C. The supernatant was removed, and free thiols were blocked with iodoacetamide (0.5 mL of a solution at 7.4 mg/mL dissolved in PBS with 0.8% SDS) for 30 min in the dark at 1200 rpm.

The resin was transferred to a spin column (Poly-prep® chromatography columns, Bio-Rad, catalog number 731-1550) and subjected to the following washes: 8 x 5 mL 0.8% SDS in PBS, 8 x 5 mL 8 M urea, 8 x 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 2000 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 1200 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 (Pierce centrifuge columns 0.8 ml, ThermoFisher Scientific, catalog number 89868) 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 HiPPR detergent removal resins (ThermoFisher Scientific, catalog number 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, Bremen, Germany).
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% H$_2$O, 2% ACN, and 0.2% formic acid and solvent B consisted of 19.8% H$_2$O, 80% ACN, and 0.2% formic acid. A 50 µm 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, Ammerbuch, Germany) to 16 cm. Samples were directly loaded onto the column resin, and the column was heated to 65° C. The peptides were separated with a 120 min gradient at a flow rate of 3220 nL/min. The gradient was as follows: 2–30% Solvent B (150 min), 30–100% B (1 min), and 100% B (9 min). Eluted peptides were then ionized using a standard coated silica tip (New Objective, Woburn, MA) 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 400-1600) 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 et al., 2008; Cox et al., 2011). Spectra were searched against the UniProt A. thaliana database (33353 sequences) and a contaminant database (245 sequences). A decoy database was generated by MaxQuant using a reversed 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 Aha (-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 Da. “Match Between Runs” and “Label-Free Quantitation” were enabled in MaxQuant. Protein and peptide identifications had an estimated false discovery rate less than 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 (Cambridge, MA). Goat anti-mouse IgG (H+L) (A-21235) and goat anti-rabbit IgG (H+L) (A-21429) secondary antibodies with conjugated Alexa Fluor were purchased from Life Technologies (Thermo Fisher Scientific, Waltham, MA). 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 non-fat dry milk in TBST for 1 h prior to incubating with antibodies in TBST. Primary antibodies [anti-ClpB1 (Ab80121, 1:5000) and anti-HSP70-5 (ab5439, 1:5000)] were incubated with their respective membranes at 4 °C overnight with 3% w/v non-fat dry milk prior to staining with the secondary antibody (1:5000) for 1 h and imaging (PMT 400 V; 50 μm pixel size). Fluorescence signals were normalized to total signal from Colloidal 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 (Figure S4A). Fluorescence intensity was plotted against total protein concentration, which shows that signals were in the linear range for accurate quantitation ($r^2 = 0.994$) (Figure S4B).

**Supplemental Data**

Supplemental Table S1: Protein Groups

Supplemental Figure S1: Concentration series

Supplemental Figure S2A: Heat map - all
Figure Legends:

**Figure 1:** Scheme of BONCAT in native plant tissues. Azidohomoalanine (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. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) was used to conjugate TAMRA alkyne to nascent proteins. Strain-promoted azide-alkyne cycloaddition (SPAAC; a biocompatible ‘click reaction’) was employed to conjugate nascent proteins to beads for enrichment.

**Figure 2:** A. Probes used in this study. L-Azidohomoalanine (Aha) is a methionine surrogate replete with an azide moiety, which renders labeled proteins amenable to cycloaddition with fluorescent alkynyl probes (5,6-TAMRA alkyne) and strained cyclooctyne reagents (DBCO-agarose). B. Time course of Aha incorporation into nascent proteins of aerial tissues in *A. thaliana* seedlings. TAMRA-alkyne was conjugated to newly synthesized Aha-tagged proteins to render them fluorescent. The gel was visualized with an excitation laser at 532 nm and an emission band pass filter at 580 nm. C. Loading control. After measuring fluorescence, the gel was stained with colloidal blue to confirm equal loading. Abbreviations: TAMRA – tetramethylrhodamine; DBCO – dibenzoazacycloc octyne.

**Figure 3:** Labeling five separate stress conditions in *A. thaliana*. A. *A. thaliana* seedlings prior to Aha pulse and stress exposure. B. Seedlings 3 h after Aha pulse (1 mM) and constant exposure to stress conditions 1-5 (defined at left of figure). C. In-gel fluorescence assay to demonstrate labeling under stress conditions. Newly synthesized proteins incorporate Aha; TAMRA-alkyne is conjugated to proteins containing Aha.
Labeling is observed under each condition. Little background signal is observed in a negative control, where plants were not exposed to Aha. D. Colloidal Blue loading control to demonstrate equal loading across lanes. Gels are representative examples of at least 3 biological replicates.

**Figure 4**: Enrichment of newly synthesized proteins for proteomics. A. Treatments used for this study. Arrowheads indicate time of introduction of Aha. B. Labeling under various conditions. C. Venn diagram of proteins identified in control conditions versus heat shock conditions. D. Volcano plot of ratios of expression levels of proteins shared between heat shock and control conditions. Proteins with higher average expression in RT samples fall on the left side of the plot, whereas proteins with higher average expression in HS samples are displayed on the right. To construct the plot, LFQ values were averaged for each condition. Then, the HS average was divided by the RT average and the Log2 value was taken. Each point represents a protein. Proteins shown in red have the GO annotation “Response to Heat”. Abbreviations: C1 – control series 1, C2 – control series 2, HS – heat shock, R – recovery, RT – room temperature, LFQ – label free quantitation, amb – ambient.

**Figure 5**: Principal component analysis of mass spectrometry results based on LFQ values. This analysis shows clear separation of control samples, heat shock samples and recovery samples. Inset shows zoom-in of controls cluster.

**Figure 6**: Partial heat map of proteins with GO annotation ‘response to heat’ found in this study. Significance of each fold change was calculated using the R package limma. Heat maps 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 heat map 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.
**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.** Heat Shock Protein 70-5 (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. Abbreviations: Rel. Fl – relative fluorescence, Cont – control (room temperature), HS – Heat Shock, Rec – Recovery.

**Supplemental Table S1**: Protein groups of control series 1 (1 mM Aha pulse; 22°C), control series 2 (2 mM Aha pulse; 22°C), heat shock (1 mM Aha pulse; 37°C) and recovery following heat stress. Temperature regimes are outlined in Figure 4A. Each condition was measured via tandem LC-MS in biological triplicate.

**Supplemental Figure S1**: Supplemental Figure: Aha concentration series. Aha labeling is essentially saturated at 1 mM (i.e., higher concentrations do not yield significant increases in incorporation).

**Supplemental Figure S2**: Heat maps show nascent protein expression patterns across conditions. To be included in the heat map, 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 (across control series 1 and series 2) was zero. Along the x-axis, control series 1, control series 2, heat shock samples and recovery samples form hierarchical clusters indicating that biological replicates are similar. **A.** All identified proteins that match the criteria above. **B.** Proteins with ANOVA p-values < 0.01. **C.** Proteins with GO annotation ‘response to heat.’ **D.** Proteins regulated by heat stress (p-value < 0.01; heat shock samples compared to control series 2) **E.** Proteins with GO annotation ‘stress response.’
Supplemental Figure S3: Immunoblotting analysis of select proteins shown in BONCAT screen to be up-regulated in response to heat stress. A. ClpB1 (HSP101, apparent molecular weight 101 kDa) and B. Heat Shock Protein 70-5 (HSP70-5, apparent molecular weight 78 kDa) 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 $0.95 \pm 0.08$ 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 measured over the entire lane.

Supplemental Figure S4: Three biological replicates show that Western Blot measurements are in the linear range. A. Three heat shock samples (biological replicates) were loaded at varying amounts of total protein to show that 10 $\mu$M is in the linear range under the exposure conditions of the blot (PMT 400 V; pixels 100 microns). B. Pixel intensities from 4A were plotted against loaded total protein. Error bars represent 1 standard deviation.

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