Optimization of Bio-Orthogonal Non-Canonical Amino acid Tagging (BONCAT) for effective low-disruption labelling of Arabidopsis proteins *in vivo*

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Running Title: Developing Bio-Orthogonal Non-Canonical Amino acid Tagging (BONCAT) for *in-vivo* labelling of newly synthesized plant proteins in plants

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ABSTRACT

Plants, as sessile organisms, require robust responses in protein synthesis to adapt to variable environmental conditions. Measurement of newly synthesized proteins has been successfully facilitated with Bio-Orthogonal Non-Canonical Amino acid Tagging (BONCAT) across a multitude of organisms. Here, we use non-canonical amino acids (NCAAs) L-azidohomoalanine (AHA) or L-homopropargylglycine (HPG) incorporation in place of methionine residues into the actively translating *Arabidopsis* proteome, allowing for that subset of proteins to be enriched for mass spectrometry quantification. Although this technique has seen occasional use in plants, optimization of the protocol to maximize functionality while minimizing organismal stress has not yet been established. Here, we provide evidence for successful implementation through the liquid immersion of seedlings in AHA or HPG-containing media that functions with significantly lower concentrations than the literature standard. Our approach splits acute exposure and incorporation phase of labelling to mitigate potential negative impacts of prolonged NCAA exposure without compromising effective enrichment capacity, and demonstrate that this results in an unperturbed growth phenotype for AHA-treated seedlings. Finally, we demonstrate the capacity of this modified approach to enrich newly synthesized proteins from the whole proteome under standard stress conditions. These improvements allow for a broader use of BONCAT technologies in molecular plant research, affording a deeper understanding of the newly synthesized proteome without negatively impacting plant health.

INTRODUCTION

The balance between protein synthesis and degradation defines proteostasis in all cells, which in turn defines the functions and capacities of all organisms. Rapid adjustment of protein levels and plasticity of the proteome is essential for survival in a changing environment, especially for plants, which are carefully tuned to their immediate environment. Defining newly synthesized proteins, or the nascent proteome, is crucial to understanding regulation of the plant cell and adaptation to environmental stressors, and can provide a quantitative measurements of the effectors produced within a cell. However, conventional strategies for labelling the newly synthesized proteome for detection via mass spectrometry (MS), such as radiolabelling, often lack depth of analysis due to abundant, older peptides obscuring detection of lower-abundance proteins of interest (Tivendale et al., 2021). Isolation of the nascent proteome would allow for a closer view of proteins lower in abundance, increasing sensitivity of analysis. To fill this niche, Bio-Orthogonal Non-Canonical Amino acid Tagging (BONCAT), has been developed to label nascent proteins with a methionine analog to allow for enrichment of newly synthesized proteins (Dieterich et al., 2006; Parker & Pratt, 2020).

Azide and alkyne groups can be irreversibly conjugated to each other via a copper-catalyzed azide-alkyne cycloaddition (CuAAC), which utilizes the efficient, biologically non-reactive click chemistry mechanism (reviewed in Chen et al., 2023). To employ this in a biological setting, unnatural methionine analogs L-azidohomoalanine (AHA, azide) or L-homopropargylglycine (HPG, alkyne) are supplied to live organisms. These non-canonical amino acids (NCAAs) are incorporated into proteins during synthesis by the relatively promiscuous methionine tRNA in a moderately methionine-depleted environment (Figure 1A), but complete methionine depletion in eukaryotes is generally not recommended, as perturbations to the proteome can be mitigated in a methionine-containing environment by limiting NCAA incorporation beyond levels required for enrichment (Bagert et al., 2014). After application, proteins are then extracted and “clicked” to the opposing azide or alkyne conjugated to biotin, effectively biotinylating newly synthesized proteins and facilitating enrichment via streptavidin resin for subsequent quantification via liquid chromatography (LC)-MS/MS. While BONCAT was initially developed for use in mammalian cells, it has since found use in a variety of organisms, such as live mice, bacteria, and archaea (Dieterich et al., 2006; Kern & Ferreira-Cerca, 2022; Lindivat et al., 2021; Saleh et al., 2019). However, unlike other organisms, BONCAT implementation in plants has not been fully realized.

In the model plant *Arabidopsis thaliana* (Arabidopsis), BONCAT method was first implemented in 2017. Here, AHA and HPG were applied to Arabidopsis seedlings through the flooding of agar plates with 1 mM AHA (Glenn et al., 2017). This yielded thousands of enriched

proteins after only a few hours of exposure time. However, subsequent endeavours found that Arabidopsis reacts metabolically to these compounds in a similar way to excess methionine, stunting growth and/or impacting plant health during prolonged exposure (Tivendale et al., 2021). Since then, only a handful of studies have utilized BONCAT in plants, and changes to the protocol have generally been limited to exposure time of the seedlings to the tags, with few studies endeavouring alternate implementations using less than 1 mM of the NCAAs (Li et al., 2023; Linster et al., 2022; Lyu et al., 2023; Xie et al., 2023; Yu et al., 2020). Together, these studies indicate that optimization of BONCAT in plants remains incomplete, as a wider variety of times and concentrations need to be tested to strike a balance between effective labelling concentrations and, ideally, minimal disturbance to the health of the seedling plants. Additionally, protocol modifications for simplicity remain unexplored, including: a) using the same biotin-conjugated clicked extract for both anti-biotin blots and enrichment for mass spectrometry to minimize variability instead of clicking to a separate fluorophore for blot detection and b) exploring the exclusivity of the azide-alkyne cycloaddition, such as eliminating the need for repeated precipitation or processing prior to the click reaction to remove metabolites (Ma et al., 2018).

Here, we present an adapted protocol for BONCAT labelling of Arabidopsis seedlings in liquid 0.5x MS medium that allows for more reliable seedling exposure to, and removal of, AHA and HPG (Figure 1B). Specifically, we demonstrate this through experimentation involving the titration of AHA and HPG as well as optimization of labelling time and approach to balance effective labelling with minimized exposure these methionine analogs. Lastly, we perform extensive seedling viability assays to ensure minimal disruption of plant growth with the modified protocol, and evaluate the newly synthesized proteome using the modified protocol under high and low salt and mannitol stress via LC-MS/MS.

RESULTS AND DISCUSSION

AHA and HPG label Arabidopsis seedlings at 50 µM or less using short application pulses

To determine concentrations of AHA and HPG suitable for application pulses, 5 d-old seedlings were acclimated to liquid medium overnight before being exposed to 0, 10, 25, 50, 100, or 250 µM AHA or HPG for 30 min (see Materials and Methods). Washed seedlings were then allowed to incorporate any remaining tag into the nascent proteome for an additional 3 h before whole plants were flash frozen and extracted. Copper-catalyzed click chemistry was then performed *in vitro* to conjugate NCAA tags to biotin, and total proteome biotinylation was

detected via immunoblot. Anti-biotin immunoblotting against untagged controls was selected as the preferred method for monitoring qualitative labelling efficiency, as whole proteome analysis may not detect AHA tags that have a mass shift similar to salt adducts of methionine (Liu et al., 2022). Although HPG appeared to have lower background labelling than AHA, the signal from AHA was observed to be higher, as above-background labelling is visible at all tested concentrations of AHA and above 25 µM for HPG (Figure 2A). Correspondingly, a concentration of 50 µM AHA or HPG was selected as an optimal concentration due to clearly visible labelling with minimal required reagent, primarily to minimize metabolic stress on the seedlings.

We then performed time-course labelling to select ideal labelling periods. Labelling periods were divided into acute exposure time (NCAA present in medium) and incorporation time (NCAA removed from medium), with NCAA proteome incorporation assessed independently. This revealed that an increase in exposure time led to an increase in labelling over the course of 24 hours for both 50 µM AHA and HPG, but above-background labelling was visible via immunoblot within the first 15 minutes (Figure 2B). Similarly, increased incorporation time with a set initial exposure of 30 min appears to increase labelling for up to 6 h after removal of tags using an initial 50 µM of either NCAA, suggesting residual NCAA may remain present in the plant's tissues after wash and removal from the medium and that the remaining reagent continues to be incorporated for several hours (Figure 2C). From this data, a 30 min exposure followed by 3 h of NCAA incorporation were selected as optimal labelling times for 50 µM of either NCAA, with a shorter acute time to mitigate potential negative effects of exposure on the seedlings. Interestingly, low concentrations of AHA (10 µM) also showed increases in labelling across longer exposure times, but labelling stagnated with prolonged incorporation (Figure 2D). Therefore, this concentration is likely too low for excess NCAA to accumulate in plant tissue prior to its removal, but may be more suitable for long-term exposure labelling, such as overnight incubations for pulse-chase assay.

Short exposure times prevent problems associated with prolonged exposure to AHA

Viability measurements of NCAA treated Arabidopsis seedlings was performed using fold-change in root length as a proxy for plant health, in order to measure the potential negative effects of AHA and HPG. Here, 5 d-old Arabidopsis seedlings were transplanted onto 0.5x MS agar plates containing AHA or HPG and allowed to grow for multiple days. All tested concentrations from 10 to 250 µM AHA and HPG decreased plant root growth across a 3-day exposure, with HPG being significantly worse than AHA, even at comparably low concentrations (Figure 3A). However, with only 24 h of exposure, 10 µM AHA did not show a significant

reduction in root growth. As this concentration shows above-background labelling within a few hours but does not continue to incorporate the NCAA after being washed (Figure 2D), 10 µM AHA may find an appropriate use in pulse-chase assays, where its capacity for 24-h acute exposure labelling could be used to label plants overnight for degradation experiments the following day.

Differing approaches have been proposed for *in vivo* AHA and HPG viability treatments in the past. Use of a flooding approach to supply 1 mM AHA or HPG to Arabidopsis seedlings, a similar step to acute exposure in this experiment, has previously been shown to negatively impact whole seedling growth and cell culture viability, in addition to perturbing metabolism over a multi-day duration, which may be related to the strict metabolic balance of methionine in plants (Tivendale et al., 2021). However, other eukaryotes demonstrate a more mixed or neutral response to exposure. For example, 2 mM AHA and 1 mM HPG have been shown to have no negative viability effects on monkey and mouse-derived cells, and experimentation on HeLa cells has shown that perturbation of the proteome in response to AHA can be mitigated as long as methionine remains present in the medium (Bagert et al., 2014; Su Hui Teo et al., 2016; Wisse et al., 2017). On the molecular level, Morey et al (2021) found mouse-derived cell lines exhibit moderate stress signals in the form of higher levels of heat shock proteins HSP70 and HSP90 when treated with 50 µM AHA, but no significant instability or increase in global protein ubiquitination during AHA treatment was observed, as well as no apoptosis or cell death that would be expected with a significant reduction in viability (Morey et al., 2021).

In contrast, Tivendale et al. (2021) found that AHA was more toxic than HPG, especially in plant cell cultures. Interestingly, bacterial studies have shown similar results to this study, with AHA being tolerable to log-phase *E. coli* at higher concentrations than HPG, where HPG reduced bacterial growth at concentrations above 2.8 µM while AHA was tolerated up to the 200 µM range (Landor et al., 2023). This effect seems to differ slightly between prototrophs and auxotrophs, where bacteria unable to synthesize essential compounds appear to be more negatively affected by HPG (Jecmen et al., 2023). Both AHA and HPG have been shown to mildly affect bacterial metabolism as well, but are not degraded as the expected products of or processed as methionine (Steward et al., 2020). Contrast in viability between these organisms and plants in different studies is likely related to difference in methods used, as well as metabolic effects.

It has been proposed that negative long-term effects of NCAAs on organism or cell growth may be irrelevant if incorporation into nascent proteins and immediate toxicity does not occur during the length of the experiment (Landor et al., 2023). All prior viability assays looked at

organism or cellular responses over a prolonged exposure time, which is not representative of the experimental conditions. To accomplish a more experimentally valid condition, we repeated viability assays with a short acute exposure in liquid 0.5x MS, followed by seedling washing and return to agar plates for 7 d to simulate prolonged incorporation time. All tested concentrations of AHA (up to 250 µM) did not exhibit reduced growth compared to control after implementation of a 30-min exposure and this protocol (Figure 3B-C), suggesting that the long-term exposure growth defects are not a result of immediate toxicity of the NCAA. In contrast, 50 µM and 100 µM concentrations of HPG showed a slight reduction in growth compared to control under the same conditions (Figure 3C). By limiting exposure to 30 min and allowing incorporation to continue for < 6 h, viability concerns of the plants can be circumvented without compromising in labelling efficiency, particularly in the use case of AHA.

AHA-labelled proteins under salt and osmotic stress

To benchmark quantitative labelling efficiency of the optimized protocol in different conditions, we applied either salt or osmotic stress during an AHA labelling period with unlabelled protein as a bead binding control and unstressed AHA-labelled seedlings as a positive control. The experiment was performed with either high stress (150 mM salt or 300 mM mannitol) or low stress (50 mM salt or 100 mM mannitol) conditions, as the effects of applied stressors may be intensified in liquid submersion. Here, we find more than 400 proteins enriched in at least one condition under high stress conditions, and more than 600 under low stress conditions, with a greater number of overlapping proteins between groups in the lower stress condition (Figure 4A). Many of these proteins are Class I hits, which we define as below detection in the negative control (Figure 4B). Additionally, using the positive control, we can dissect stress specific responses. Network analysis of the hits exclusive to salt and mannitol stress, even in the lower stress conditions, resolves multiple protein networks related to abiotic stimulus response (Figure 4C). Gene ontology enrichment analysis also resolved numerous abiotic stress response biological processes in the stress conditions than versus the control, including biological processes such as wax biosynthesis and water-related regulation (Figure 4D, highlighted).

SUMMARY

In this study, we established a workflow for the use of AHA and HPG to label and enrich the newly synthesized proteome of Arabidopsis in less than 4 h without reducing seedling viability. Although 50 µM AHA was selected as the optimal labelling concentration, the protocol is flexible and modifiable based on need, and modified protocols can be tracked for efficiency at multiple

stages via blotting from the same clicked sample that is used for enrichment. Enriched samples show clear definition between stress and unstressed conditions in relatively short periods. As a technique that allows for isolation of the newly synthesized proteome, establishing a BONCAT workflow in Arabidopsis that mitigates negative plant responses unveils outstanding opportunities for applications where enrichment of a subsect of the proteome is required for sufficient depth of analysis. Lastly, the potential for NCAA tag incorporation at concentrations substantially below literature standard has positive implications for the use of this technique in plants moving forward.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis thaliana seeds (Col-0) were liquid sterilized by immersion in 70% (v/v) ethanol for 2 min followed by 30% (v/v) bleach for 10 min. Sterilized seeds were vernalized in H_2O in the dark at 4°C for 3 d. Germination plates were prepared using half-strength Murashige & Skoog (MS) basal salts, pH adjusted to 5.8 with aqueous KOH, with 0.08% (w/v) agar.

For click chemistry, vernalized seeds were stratified on plates and allowed to grow with plates positioned vertically in 24-h light at 22°C for 5 days. Approximately 100 mg of 5 d-old seedlings were immersed in autoclaved half-strength MS media in 24-well plates and placed under constant light for 16 hours to acclimatize. MS media was removed and replaced with 50uM filter sterilized L-azidohomoalanine or L-homopropargylglycine (Vector Labs, CCT-1066 and CCT-1076) from 0.1M stocks dissolved in H_2O and diluted in 0.5x MS media. Seedlings were then incubated for 30 minutes of exposure time in constant light before being washed once in 0.5x MS and incubated in constant light in 0.5x MS for 3 hours of “incorporation” time. Labelled seedlings were removed from media, rinsed in 0.5x MS, and quickly touch dried before being flash frozen in liquid N_2 and stored at -80°C until subsequent steps.

For root length assay of reagent toxicity, seeds were sown individually on 0.5x MS agar plates positioned vertically in dark boxes with seeds at the light interface to emulate roots growing in the dark and shoots growing in the light. Seedling growth conditions consisted of a 12 h photoperiod, 22°C and 100 µmol/m^{2}s light. After 5 d for the long-term reagent exposure, seedlings were transplanted along the same light/dark interface to 0.5x MS plates containing 0, 10, 25, 50, or 250 µM AHA or HPG. For the short-term exposure, seedlings were placed into liquid 0.5x MS overnight to equilibrate, then immersed for 30 minutes in 0.5x liquid MS containing 0, 10, 25, 50, or 250 µM AHA, before being washed in 0.5x MS and re-plated on to vertical plates. Root length was recorded for 7 d using digital calipers, and fold change in

individual seedling root length relative to day 0 (transplanting day) was plotted. Recording of root length stopped after day 3 for the long-term exposure due to statistically significant differences between all concentrations relative to controls.

Optimization of plant labelling

To determine optimal concentrations of label, variable concentrations of 10, 25, 50, 100, or 250 µM of AHA or HPG were applied as above. Optimal concentrations for labelling were chosen based on a combination of intensity of labelled protein present on streptavidin immunoblots and minimal observed growth retardation on 0.5X MS agar containing the same concentration of label. For time-course experimentation, acute exposure times were varied between 15 min, 30 min, or 1, 2, 4, or 22 h. Incorporation times were varied between 30 minutes or 1, 2, 3, 6, or 24 h. Optimal incubation times were chosen based on minimal required times to achieve effective labelling at the desired concentration as visualized via immunoblot, and verified to cause insignificant or no growth inhibition to the seedlings based on viability studies.

Protein extraction and quantification

Flash frozen tissue was ground using Geno/Grinder (SPEX SamplePrep) for 30 s at 1200 rpm with 3mm glass beads and extracted in either 50mM HEPES (pH 7.5) with 1% (w/v) SDS or 50 mM Tris (pH 8.0) with 1% (w/v) SDS, at a ratio of 1:2 w/v ground tissue to extraction buffer. Following optimization, HEPES was selected as the extraction buffer for all samples due to better click labelling observed. Samples were heated at 95°C for 10 min and 1000 rpm on Eppendorf ThermoMixer F2.0, cooled, and centrifuged at 16,000 xg for 10 min at room temperature to remove tissue particulates. Protein amounts were quantified using Bradford assay with samples diluted to 0.2% (w/v) SDS against BSA controls.

Click chemistry reaction

A total of 250 µg of protein from each sample was diluted in extraction buffer with 1% (w/v) or no SDS to a volume of 346 µL ensuring that a final concentration (in 400uL total) of 0.5% (w/v) SDS to maintain protein solubility in solution. To each reaction, the following was added: 8uL of 50 mM tris(2-carboxyethyl)phosphine (TCEP)-HCl (Sigma; diluted from 1 M stock in H_2O same day of use), 30 µL of 1.7 mM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Vector Labs CCT-1061; in 20% (v/v) dimethyl sulfoxide (DMSO) and 80% (v/v) tert-butanol, stored at room temperature as in Ma et al., 2018), 8 µL of CuSO4 (Sigma; in H_2O, stored at -20°C), and 8 µL of 5mM biotin-PEG3-azide (Vector Labs CCT-AZ104; for HPG samples, in DMSO) or biotin-

PEG4-alkyne (Vector Labs CCT-TA105; for AHA samples, in DMSO). Click reactions were allowed to proceed for 1 h at room temperature, shaking at 700 rpm and protected from light. Reactions were then quenched via chelation with 20 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0, stored at 4°C).

Validation of click chemistry labelling was determined via streptavidin immunoblot. Here, 6 µg of protein was run on 10% SDS-PAGE gels then transferred to 0.45 µM polyvinylidene fluoride (PVDF) membrane semi-dry over 45 min at 15 V. Blots were blocked in 5% fat-free milk in Tris-buffered saline (TBS) for 1 h, at which point HRP-conjugated streptavidin diluted 1:2000 in Tris-buffered saline with 0.1% Tween-20 (TBST) was added for an additional hour. Membranes were washed in TBST and visualized using Clarity Western ECL Substrate. Colloidal Coomassie Blue-stained gels were used as loading controls.

Enrichment of clicked proteins

Excess biotin was removed from clicked samples using Cytiva PD Spintrap G-25 columns. Bead binding comparison was done using Invitrogen streptavidin-C1 magnetic beads and Cytiva streptavidin Sepharose beads; all depicted experiments proceeded using non-magnetic Sepharose beads. All beads were initially washed 3 times in 50mM HEPES, pH 7.5, for 1 min while rotating at room temperature. For all steps, supernatant was removed from magnetic beads by adhering beads to a magnetic rack, and from other types of beads by centrifugation for 1 minute at 600 xg. Up to 200 µg of flow-through protein from the Spintrap columns was loaded onto equilibrated streptavidin beads and allowed to bind while rotating for 16 hours at 4°C. The supernatant was removed and retained at -20°C to assess capture efficiency.

Capture efficiency was checked before digestion via streptavidin-detected dot blot comparison of clicked protein, Spintrap column flow-through, and supernatant after overnight bead binding. After confirmation of a signal reduction of more than 70% in the supernatant after binding, beads were washed sequentially with the following solutions at 1mL each: 50 mM HEPES pH 7.5 (2x washes), 1 M potassium chloride, ice-cold 100 mM sodium carbonate (2x washes), 2 M urea in 50 mM Tris-HCl pH 8.0 (2x wash), and 50 mM triethylammonium bicarbonate (TEAB) in HPLC-grade water (3x washes). All washes were completed at room temperature with rotation for 3 min.

Peptides were generated through on bead digestion using trypsin diluted 1:100 in 50 mM TEAB overnight at 37°C; the supernatant was removed before the beads were washed with 50 mM TEAB and the supernatant pooled with previously extracted peptides. The extract was reduced with 10 mM DTT at 95°C for 5 min while shaking at 450 rpm, alkylated with 30 mM

iodoacetamide (IA) at room temperature for 30 min, and the reaction quenched with 10 mM DTT for 10 minutes. Samples were then acidified to 0.5% (v/v) TFA to a pH of less than 2 and centrifuged at 16,000 xg for 1 min before the supernatant was removed and dried down. Peptides were re-suspended in 3% (v/v) acetonitrile / 0.1% (v/v) formic acid for desalting via ZipTip (Sigma cat. no. ZTC18S960) before being dried and stored at -80°C. Desalted peptides were the re-suspended in 3% (v/v) acetonitrile / 0.1% (v/v) formic acid for mass spectrometry analysis.

LC MS/MS and Data Analysis

Peptides were analyzed on a FAIMSpro mounted Fusion Lumos Orbitrap mass spectrometer (Thermo Fisher Scientific) in data independent acquisition (DIA) mode. Re suspended peptides were injected using Easy-nLC 1200 system (LC140; Thermo Fisher Scientific) and an Acclaim PepMap 100 C18 trap column (Cat# 164750; Thermo Fisher Scientific) followed by a 50 cm Easy-Spray PepMap C18 analytical column (ES903; Thermo Fisher Scientific) warmed to 50 °C. Peptides were eluted at 0.3 µl/min using a segmented solvent B gradient of 0.1% (v/v) formic acid in 80% (v/v) acetonitrile (A998, Thermo Fisher Scientific) from 4 to 41% solvent B (0–60min). The FAIMSpro was used with a fixed gas flow of 3.5 L/min with a 3-CV setting of -30, -50, -70. A positive ion spray voltage of 2.3 kV was used with an ion transfer tube temperature of 300 °C and an RF lens setting of 40%. Direct DIA acquisition was performed as previously described (Mehta et al., 2022). Full scan MS^1 spectra (350–1400 *m*/*z*) were acquired with a resolution of 120,000 at 200 *m*/*z* with a normalized automatic gain control (AGC) target of 100% and IT set to automatic. Fragment spectra were acquired at resolution of 30,000 across 28 38.5 *m*/*z* windows overlapping by 1 *m*/*z* using a dynamic maximum injection time and an AGC target value of 2000%, with a minimum number of desired points across each peak set to 6. Higher-energy collisional dissociation fragmented was performed using a fixed 27% fragmentation energy. Downstream data analysis of dDIA acquisitions was performed using Spectronaut ver. 19 (Biognosys AG) with default settings as previously described (Mehta et al., 2022), with all searches made against a custom-made decoy (reversed) version of the Arabidopsis protein database from Araport 11 (27,533 protein encoding genes; ver. 2022-09-14). Briefly, search parameters included the following: trypsin digest permitting two missed cleavages, fixed modifications (carbamidomethyl (C)), variable modifications (oxidation (M)) and a peptide spectrum match, peptide and protein false discovery threshold of 0.01.

Bioinformatic Data Analysis

Gene Ontology (GO) enrichments were performed using the Ontologizer (http://ontologizer.de/; Bauer et al., 2008) using a Benjamini-Hochberg parent-child union with an analysis cutoff of $p > 0.01$, and visualized in R 4.5.0. Significantly changing proteins (Log2FC of MS2 spectra > 0.58 over negative control) were pooled with Class I protein hits (absent in MS2 of negative controls) to form the foreground with a background of all identified proteins (Supplemental Table 1). A subset of the same dataset that was below threshold abundance in both positive and negative controls was used for network analysis of stress-exclusive proteins via the StringApp plugin for Cytoscape v. 3.10.3, with an edge score cutoff of 0.5 (Doncheva et al., 2019, https://cytoscape.org/). The STRING Enrichment within StringApp was used to assign outlines based on gene ontology. Non-network visualizations were produced using the *ggplot2* R package, Biorender (https://www.biorender.com/), GraphPad Prism v. 8.4.3 (https://www.graphpad.com/), and Affinity Designer v. 1.10.5 (https://affinity.serif.com/en-us/designer/).

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AUTHOR CONTRIBUTIONS

Nicholas Hassan: Investigation, Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing—original draft. *Shelly Braun*: Writing—review & editing, Conceptualization. *Mohana Talasila*: Formal analysis, Data curation, Visualization. *Richard Fahlman*: Supervision, Writing—review & editing, Material Resources *R. Glen Uhrig*: Supervision, Conceptualization, Writing—review & editing, Funding acquisition.

DATA AVAILABILITY

Raw data have been deposited to the ProteomeExchange Consortium (http://proteomecentral.proteomexchange.org) *via* the PRoteomics IDEntification Database (PRIDE; https://www.ebi.ac.uk/pride/) partner repository with the data set identifiers PXD064210

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FIGURES

Figure 1. Visualization of BONCAT technology. A) Incorporation mechanism of AHA and HPG into the nascent proteome.

Figure 2. Streptavidin-HRP anti-biotin blots of biotin-clicked Arabidopsis whole lysate samples.

Figure 3. Viability assays of Arabidopsis seedlings exposed to methionine analogs.

Figure 4. Summary mass spectrometry data for 50μM AHA-labelled 3-hour salt or mannitol stressed Arabidopsis seedlings.

SUPPLEMENTAL TABLES

Supplemental Table 1. Enriched AHA-treated low and high salt and mannitol stress proteomics data.

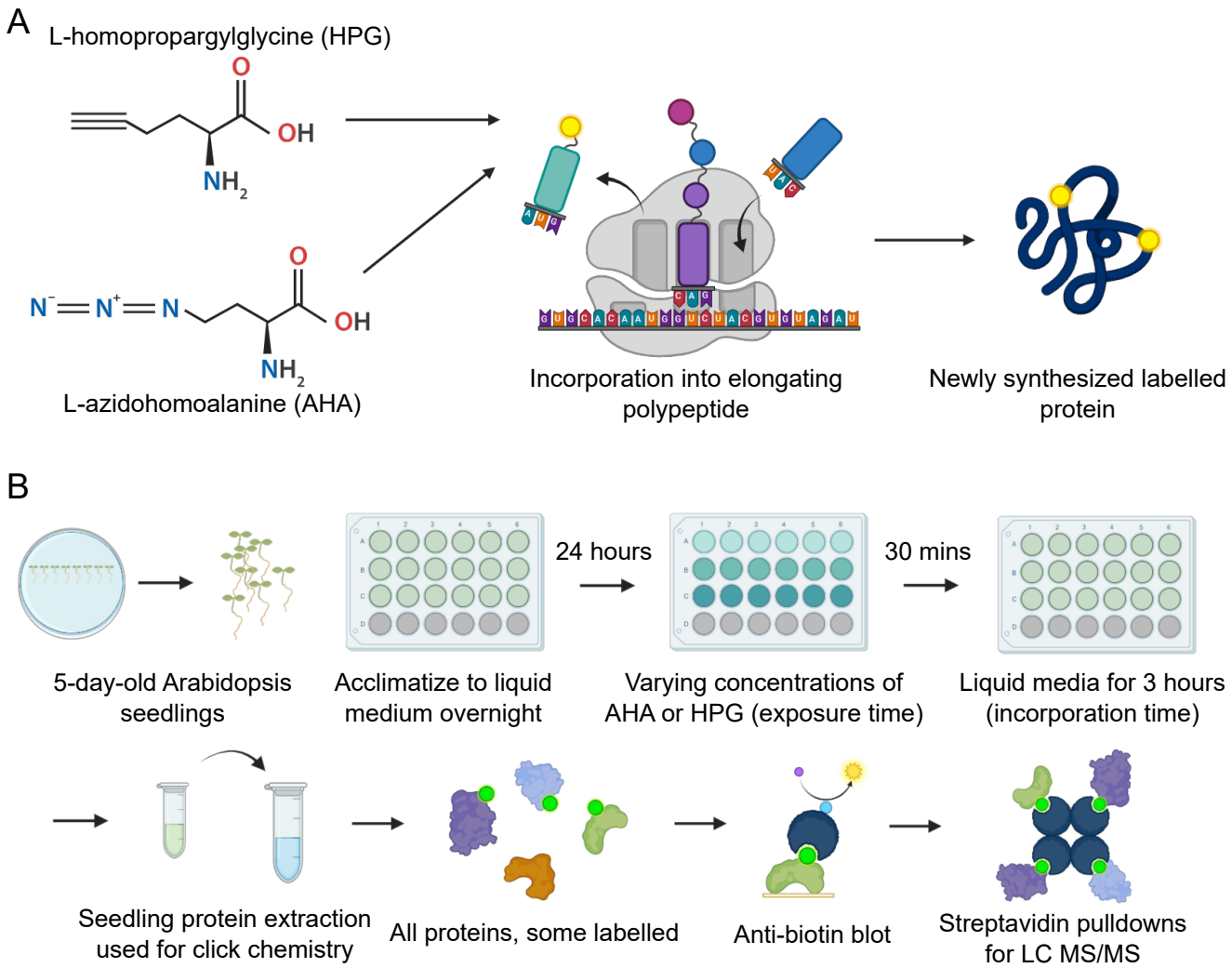

Figure 1. Visualization of BONCAT technology. A) Incorporation mechanism of AHA and HPG into the nascent proteome. B) Workflow for click labelling. Whole seedlings are acclimatized to liquid 0.5X MS media for 24 hours before treatment with AHA or HPG. Acute exposure time in the modified protocol is limited to 30 minutes, followed by 3 hours of incorporation time. Protein is extracted from seedlings for click chemistry in vitro, followed by visualization via anti-biotin blot to determine qualitative labelling before streptavidin enrichment for LC-MS/MS.

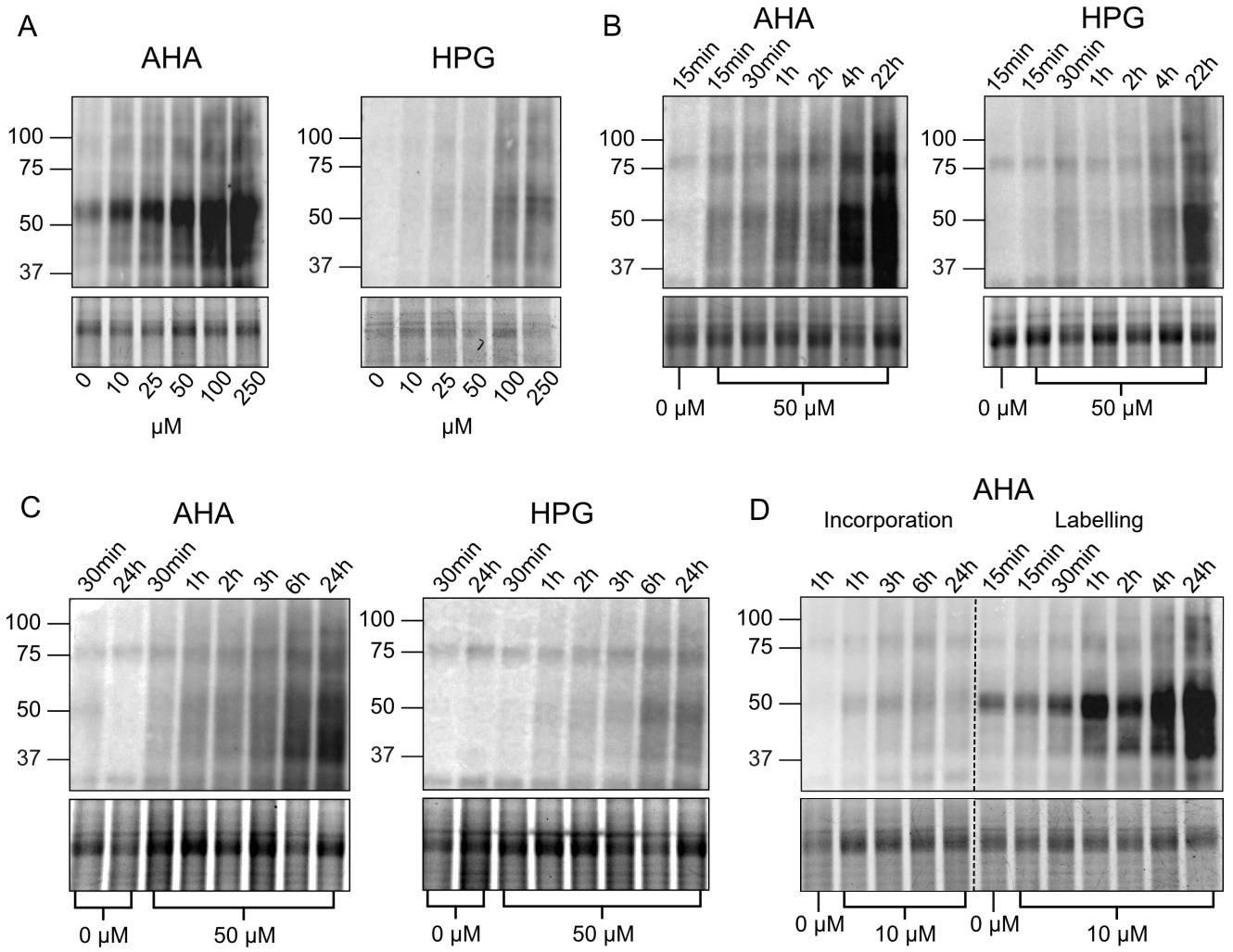

Figure 2. Streptavidin-detected anti-biotin blots of biotin-clicked Arabidopsis whole lysate samples. Beneath each blot is a cropped segment of a Coomassie Blue gel loading control. Each experiment was performed in triplicate, but single replicates are shown for simplicity. A) Titration of AHA and HPG concentrations with a 30-minute acute exposure and 3-hour incorporation time. B) 50μM AHA and HPG tested with exposure times varying from 15 minutes to 22 hours, all followed by a 3-hour incorporation. C) 50μM AHA and HPG tested with incorporation times varying from 30 minutes to 24 hours, each preceded with a 30-minute exposure time. D) Low concentration AHA (10μM) test of varying incorporation times with a 30-minute exposure (left) or varying exposure times with a 3-hour incorporation (right).

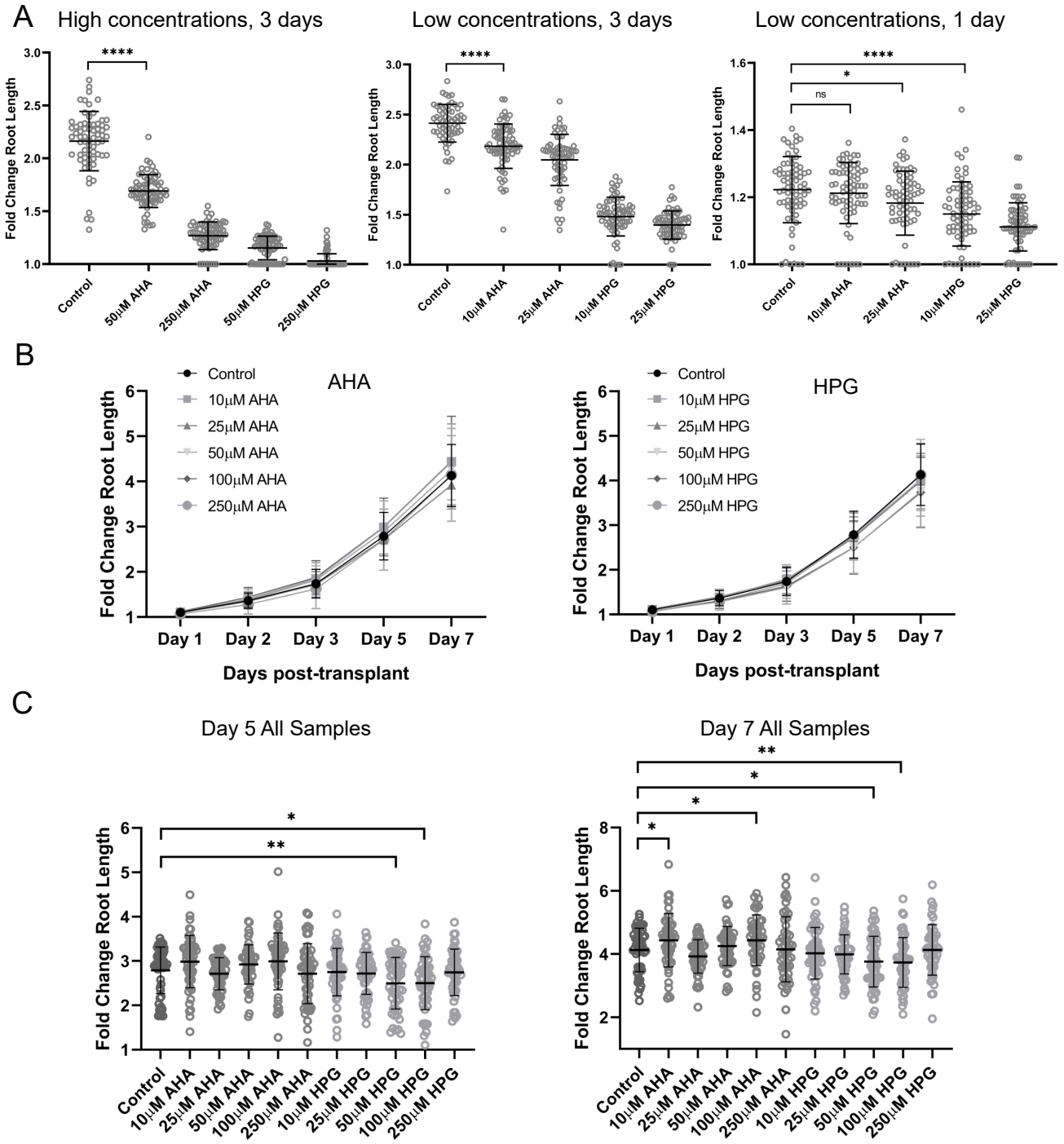

Figure 3. Viability assays of Arabidopsis seedlings exposed to methionine analogs. Average fold change in root length of seedlings with standard deviation error bars is plotted, and t-tests between experimental and control groups were performed with p-value significance <0.05 shown. A) n = 72 5 d (days)-old seedlings per condition were transplanted to agar plates containing AHA or HPG for 1-3 d to simulate extensive exposure times. High concentrations after 3 days (left), low concentrations after 3 days (middle) and low concentrations after 24 hours (right) are shown. B) n = 54 seedlings per condition were acclimatized to liquid media for 24 h, then exposed for 30 min in AHA- or HPG-containing liquid media before being returned to solid medium and grown for 7 d. C) Snapshot of days 5 and 7 for all conditions from summarized data in 2B to show significant changes.

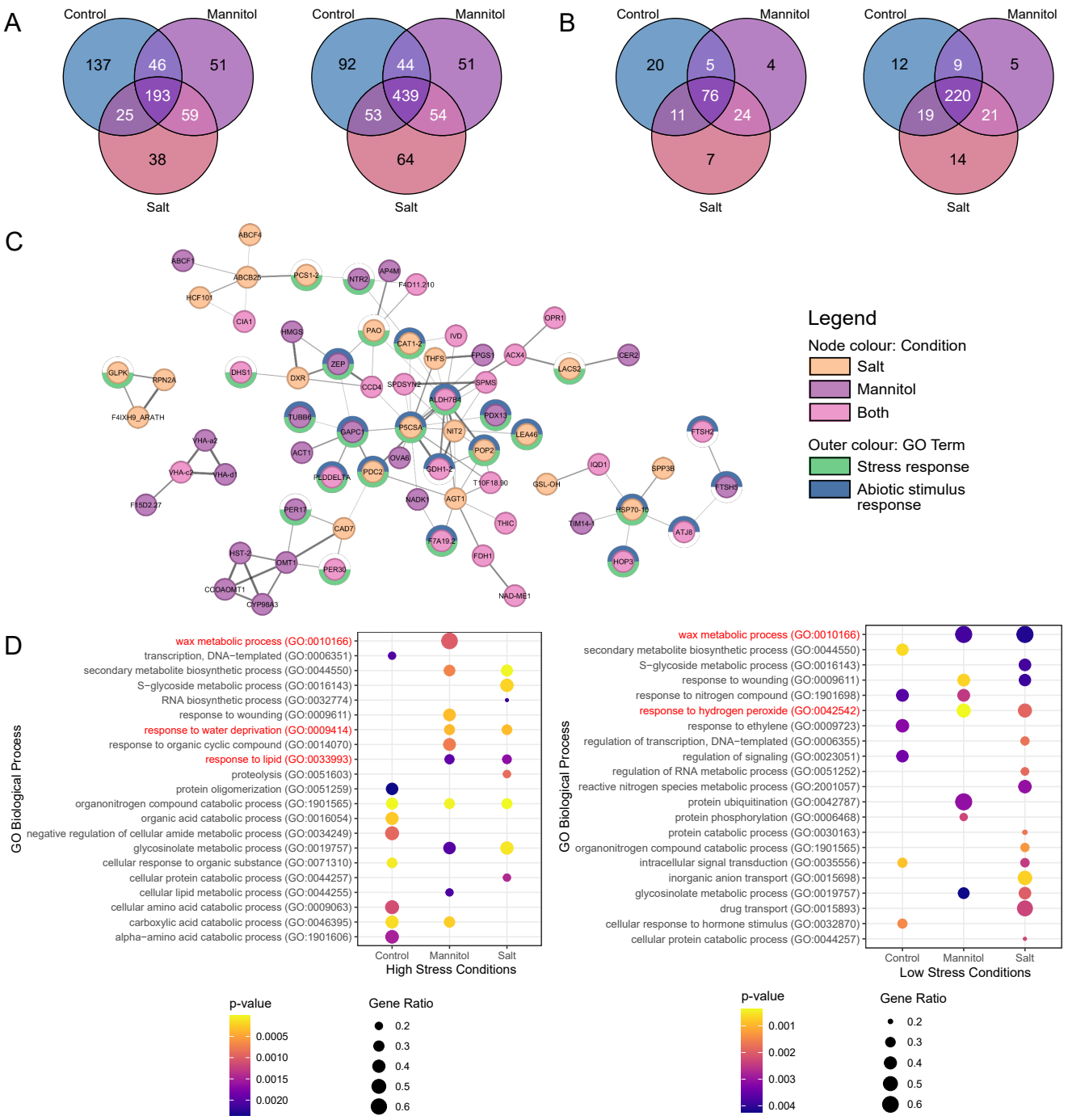

Figure 4. Summary mass spectrometry data for 50µM AHA-labelled 3-hour salt or mannitol stressed Arabidopsis seedlings. A) Venn diagrams of valid (>1.5X FC or below detection in negative control) protein groups between positive control (AHA treated, unstressed) and stress conditions in either high stress (150mM salt and 300mM mannitol, left) or low stress (50mM salt, 100mM mannitol, right) conditions. B) Exclusively Class I hits from 1A that are absent in negative controls. C) STRING network of valid protein hits enriched in the low stress salt and/or mannitol conditions that were below detection or below threshold in both positive and negative controls. Only nodes with >2 edges were kept. Increasing line thickness is edge score ranging from 0.5-1. D) Gene ontology biological processes (Benjamini-Hochberg parent-child union) for high stress (left, $p > 0.0025$) and low stress (right, $p > 0.005$) conditions. All proteins enriched in one condition over negative controls were included. Highlighted in red are terms directly related to stress response.