ASSESSING STRESS TOLERANCE IN SMALL HEAT SHOCK PROTEIN MUTANTS IN *ARABIDOPSIS THALIANA*

An Honors Thesis Presented

By

PARTH PATEL

Approved as to style and content by:

Elizabeth Vierling
Chair

Minsoo Kim
Committee Member

Patrick Karl Treffon
Committee Member

Elizabeth Vierling
Honors Program Director

ABSTRACT

Molecular chaperones are proteins found in virtually every organism and are essential to cell survival. When plants are heat stressed, they upregulate and downregulate multiple genes, many of which are associated with the heat shock response. Small heat shock proteins (sHSPs) are one class of molecular chaperones that are upregulated during heat shock. They are proposed to act as the first line of defense by binding to heat sensitive proteins and preventing their irreversible aggregation. Many details of sHSP function remain to be discovered, and exactly what proteins they protect is unresolved. In addition to cytosolic sHSPs found in other organisms, plants also produce sHSPs that are targeted to chloroplasts and mitochondria. The four sHSPs that are found in organelles in *Arabidopsis thaliana* are HSP23.5-M/C, HSP23.6-M/C, HSP25.3-P, and HSP26.5-MII. In this study, the heat tolerance of knockout mutants of these different organelle-localized sHSPs, including single, double, and triple knockouts was assessed through a hypocotyl elongation assay, a hypocotyl elongation assay for thermomemory, and an assay with light grown seedlings. The hypocotyl elongation assay indicated a phenotype for mitochondria- localized sHSPs as their absence showed reduced hypocotyl elongation following heat stress. The hypocotyl elongation assay for thermomemory showed no phenotype for any sHSP knockout mutant. The light grown seedling assay exhibited too much variability in the response such that no conclusions could not be drawn, however the parameters for future assays were determined. Understanding the phenotypes of these sHSPs will bring us closer to defining their mechanism of action and the mutants will provide a platform for further studies of sHSP structure and function.

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INTRODUCTION

Introduction to molecular chaperones

Molecular chaperones, such as heat shock proteins (HSPs), are proteins found in virtually every organism and are essential to cell survival. Because the structure of a protein is critical to its function, molecular chaperones play an important role in cellular protein homeostasis by helping proteins fold, retain their shape, and even by unfolding and reactivating proteins that have aggregated and lost function. Some chaperone proteins are normally expressed at basal levels and become more highly expressed in times of stress, especially heat stress, which causes protein unfolding. The small HSPs (sHSPs) are one class of molecular chaperones. They are proposed to act as the first line of defense by binding to heat sensitive proteins and preventing their irreversible aggregation. sHSPs then present the bound, heat-sensitive proteins to other HSPs that can help reactivate them through ATP-dependent mechanisms (Haslbeck & Vierling, 2015). The mechanism of substrate capture by sHSPs is not well understood, although there are some hypotheses as to how they achieve this feat. What proteins are sHSPs substrates is also not well known, but they are thought to bind a wide range of different proteins. Like all HSPs, sHSPs are found in all kingdoms of life, but are uniquely diverse in land plants and are likely critical to plant survival.

Arabidopsis sHSPs

Plants express a high level and diversity of sHSPs and their corresponding mRNAs in response to elevated temperature, oxidative stress, and other types of stresses, as well as at certain points of development. In plants, sHSPs also accumulate in every cellular organelle – the nucleus, endoplasmic reticulum, peroxisomes, mitochondria and chloroplasts, as well as in the cytoplasm (Basha et al., 2012). Plants are immobile and cannot escape environmental stresses,

and so, there may have been significant evolutionary pressure to retain and select for sHSPs, eventually leading to the diversity of sHSPs seen in higher plants. In comparison to bacteria and humans, plants can generate over 20,000 transcript copies per cell and synthesize a large number of HSPs that are between 15 and 42 kDa in size (Santhanagopalan et al., 2015). The first sHSP sequences were obtained from soybeans in 1985 and were recognized to be homologous to sHSPs that had been already characterized in *Drosophila*, *Cenorhabditis elegans* and *Xenopus* (Nagao et al. 1985). *Arabidopsis thaliana* has 19 different sHSPs, while humans only have 10 (Haslbeck et al., 2005 as cited by Sedaghatmehr et al., 2016). Many more sHSPs from plants and other organisms have been characterized since the mid-1990s. Here, the focus will be on the sHSPs in plant mitochondria and chloroplasts, which are the major sites of energy generation in plant cells. It is therefore particularly important for molecular chaperones to maintain protein homeostasis in these organelles.

Structure of sHSPs

Key information to understanding proteins comes from their structures. sHSPs are dynamic, oligomeric proteins, ranging from 12 to >32 subunits, and solving their crystal structures has been difficult. However, an important sHSP structure has been solved, dodecameric wheat HSP16.9 (Van Montfort et al., 2001). It is one of the only complete eukaryotic sHSP X-ray structures at high resolution (Santhanagopalan et al., 2015). sHSPs are characterized by their structural similarities, consisting of a N-terminal domain (anywhere from 24-84 amino acids), an α-crystallin (ACD) domain (90-100 amino acids), and a C-terminal extension (0-18 amino acids). Although the N-terminal domain is variable in length and sequence, some motifs can be recognized. The disorder of many N-terminal domains in various

crystal structures, as well as dynamic features of the N-terminal arms as observed by NMR, have led to the suggestion that they are intrinsically disordered (Uversky and Dunker, 2010). This and other data has led some researchers to propose that the N-terminal arms are a major substrate binding domain (Santhanagopalan et al., 2015). In contrast, the ACD and C-terminal extensions are largely conserved. The ACD comprises a seven-stranded beta sheet with a IgG-like fold that assembles into dimers that are a conserved substrructure of sHSP oligomers. The C-terminal extension that follows the ACD contains a conserved I/V/L-x-I/V/L motif (IXI motif) that was first recognized in 1998 and is found in a majority of sHSPs (de Jong et al., 1998).

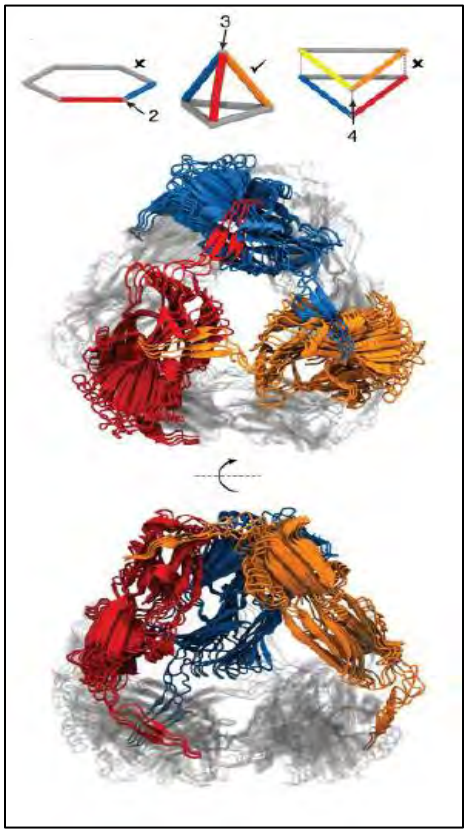

Figure 1. Geometry of the wheat HSP16.9 dodecamer as described by Santhanagopalan et al., 2018. The most current structure suggests a tetrahedral model. The three dimers are colored (blue, orange, red) and three are rendered in gray.

The importance of the IXI motif lies in the observation that it makes a significant contact that links sHSP dimers into higher order oligomers. The whole C-terminal extension has also been seen to adopt different angles in relation to the ACD to generate oligomers of different sizes and geometries (van Montfort et al., 2001). sHSPs are relatively small proteins, but they assemble into dimers (which requires a strand swap from one monomer to the other) and then into oligomers that can have 12 to more than 24 subunits (Delbecq and Klevit, 2013). The oligomers have been observed in other mass spectrometry experiments.

Located at the N-terminal of specific plant sHSPs is a sequence that provides the information that targets the sHSP to the correct cellular compartment. Many of the crystal structures for sHSPs do not include complete structural information on the N-terminal domain due to a high amount of disorder. Nonetheless, the structure of the protein and its quaternary interactions provide valuable insight to the potential mechanisms of these molecular chaperones as recent publications indicate that the three-dimensional quaternary structure of plant cytosolic sHSPs is a tetrahedron formed by six sHSP dimers (Figure 1; Santhanagopalan et al., 2018).

Class I and Class II sHSPs

Extensive biochemical characterization is available for two classes of plant sHSPs, Class I (CI) and Class II (CII), both of which are cytosolic sHSPs and are induced by heat stress, together accumulating to over 1% of the total cell protein within a few hours (Derocher et al., 1991). It is thought that the CI and the CII proteins evolved through gene duplication over 400 million years ago (Waters and Vierling, 1999). Both classes of proteins form dodecameric oligomers, but the two classes do not form heterooligomers; rather they will only heterooligomerize with proteins from the same class. Previous experiments also show that after heat stress, there were significantly more proteins bound to CI sHSPs compared to CII sHSPs. Additionally, when heat stress does occur, CI sHSPs are more tightly associated with translation factors and related proteins. In that same experiment, a large reduction of either the CI or CII sHSPs was enough to compromise the ability of seedlings to recover from extended heat treatment after acclimation (McLoughlin et al., 2016).

Proposed mechanism of action of sHSPs

While many molecular chaperones are ATPases, sHSPs are ATP-independent molecular chaperones that are thought to prevent irreversible aggregation of stress sensitive proteins (Santhanagopalan, et al., 2015). They are able to bind up to an equal weight of substrate protein. They are known to interact with ATP-dependent HSPs to restore an inactive protein substrate to its original and active state. It has been proposed that the sHSP oligomers act as "reservoirs" of a dimeric sHSP unit (considered to be the active unit). The dimeric units become available to stressed cellular proteins upon activation of the sHSP (Figure 2). Stress is suggested to activate the sHSPs by shifting the equilibrium to the dimeric form, which binds unfolded or misfolded proteins and prevents further unfolding or aggregation. The equilibrium between oligomers of sHSPs and sHSP dimers has been shown by several experiments (Santhanagopalan, et al., 2015). Dissociation into dimers is assumed to increase surface area, making regions that are normally buried in the sHSP oligomers available for binding substrates. The interaction between protein substrates and sHSPs are considered to occur through exposed hydrophobic surfaces (Basha et al. 2012; Lee et al. 1997; Van Montfort et al. 2001a). However, the details of how sHSPs interact with substrate, the extent to which sHSPs exhibit substrate specificity and why they have a higher affinity for denatured or misfolded proteins, as well as the other functions that sHSPs may serve remain largely unknown (Haslbeck & Vierling, 2015). The sizes of the sHSP-substrate complexes that form after heat stress have been observed to be dependent on the concentration of sHSPs relative to substrate. *In vitro* experiments showed that when sHSPs are abundant, sHSP-substrate complexes are smaller, likely because there is less self-aggregation of substrate due to higher availability of sHSP to form contacts with substrate.

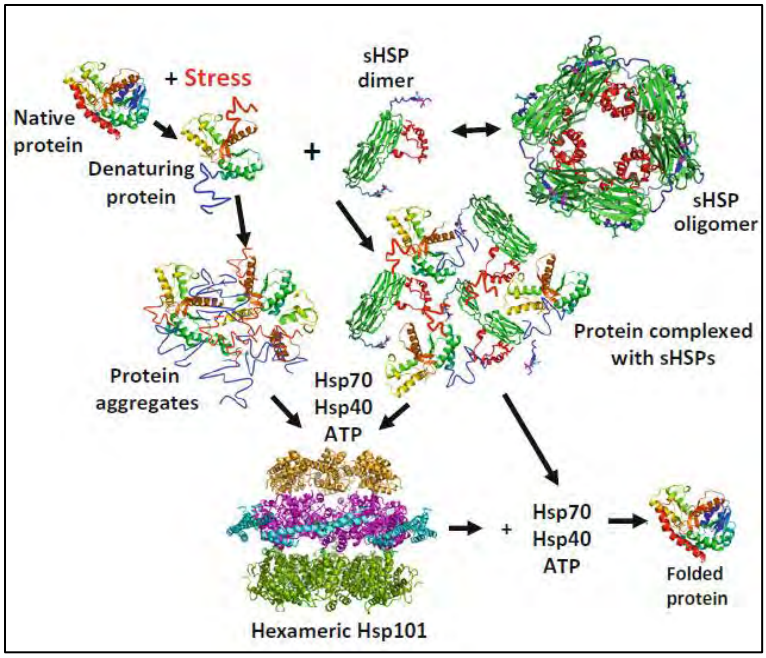

Figure 2. Model for sHSP mechanism of action. It has been proposed that the sHSP oligomers act as "reservoirs" of a dimeric sHSP unit (considered to be the active unit). The dimeric units become available to stressed cellular proteins upon activation of the sHSP. Stress is suggested to activate the sHSPs by shifting the equilibrium to the dimeric form, which binds unfolded or misfolded proteins and prevents aggregation or further aggregation (from Santhanagopalan et al., 2015**)**.

Conversely, when sHSPs are limiting, sHSP-substrate complexes are larger because there is not enough sHSP to block the self-interaction of denaturing proteins (Friedrich et al., 2004). sHSP-substrate complexes are a few hundred to a few thousand kDa and do not release the substrates because sHSPs cannot bring about disaggregation on their own. Because of this, addition of sHSP after aggregation did not decrease the size of the sHSP-substrate complexes. Also hundreds of sHSP:substrate stoichiometries were observed in complexes, which suggested that the sHSPs capture substrates without a specific binding site, maybe due do different degrees of substrate unfolding (Stengel et al., 2010). Related work found that most sHSP-substrate complexes have an even number of sHSP monomers, supporting the sHSP dimer as the major substrate binding species (Stengel et al., 2012). However, other experiments have shown that the

dimeric interface is also labile and that the dimers dissociate under stress conditions. For this reason, sHSP-substrate complexes carrying an odd number of sHSP monomers have also been observed, although to a lesser extent than those with even numbered species.

Chloroplast and mitochondrion localized sHSPs

When Arabidopsis plants are heat stressed, certain sHSP transcripts become highly elevated. These sHSPs include HSP23.5-MI/C, HSP23.6-MI/C, HSP25.3-P (the latter also (formerly) known as HSP21) and HSP26.5-MII (shown in Figure 3). HSP25.3-P localizes only to the chloroplast. HSP26.5 localizes only to the mitochondrion. The other two sHSPs, HSP23.5 and HSP23.6, localize to both the mitochondrion and the chloroplast under heat stress conditions. These four sHSPs are not detected in western blot analysis when plants are grown at room temperature. When the plant is heat stressed, the proteins become apparent and can be detected by corresponding antibodies.

There have been many experiments performed on HSP25.3-P, the sHSP that localizes in the chloroplast (and root and other plastids) and is found in all land plant species. It is characterized by a unique amphipathic, Met-rich motif located in the N-terminal domain that is conserved in almost all chloroplast sHSPs, but not found in other sHSPs (Chen and Vierling, 1991). An early study suggested that HSP25.3-P plays a role in protecting photosystem II against heat stress (Heckathorn et al., 1998), as well as other types of stresses. However, despite various studies, the molecular mechanism of action remains largely unknown. We hypothesize that HSP25.3-P interacts with proteins that are in the chloroplast and helps maintain homeostasis in this compartment under stressful conditions.

Besides localizing in the mitochondrion, not much is known about HSP26.5-MII. The reason it is known is because it shows up in *Arabidopsis* proteomic studies. It is thought to interact with mitochondrial proteins and to maintain homeostasis of these proteins during heat stress. One hope with the experiments in this thesis is better characterization and insight into HSP26.5-MII function.

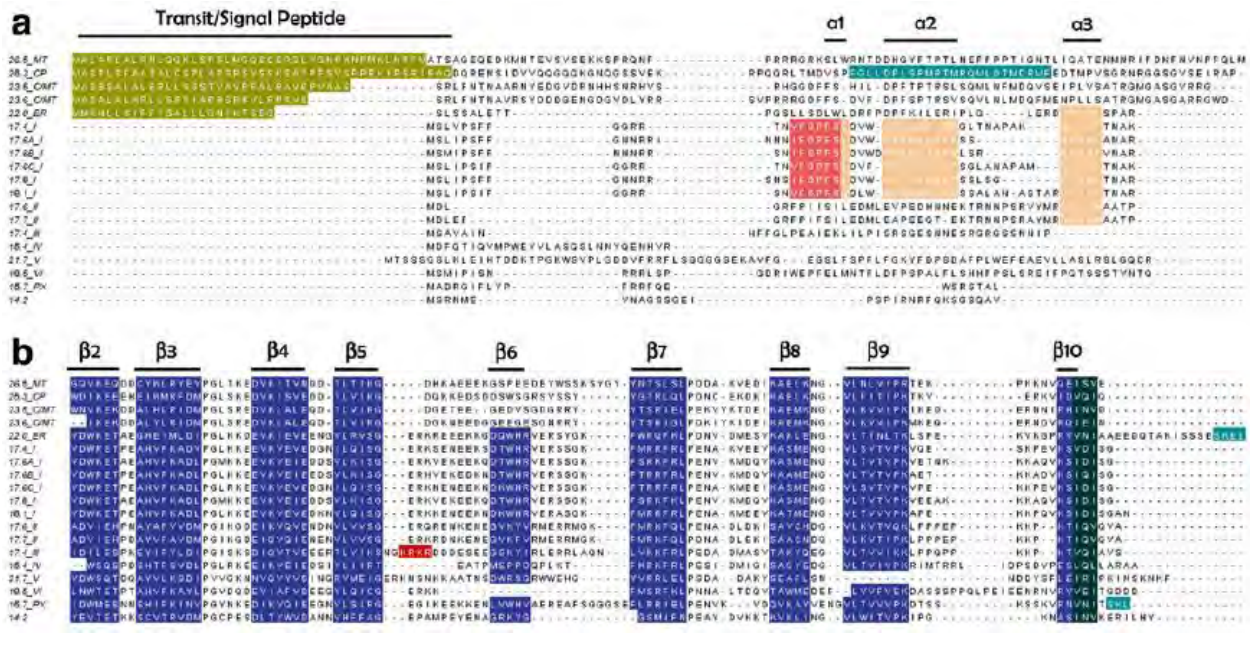

Figure 3. Sequence alignment of 19 sHSPs found in *Arabidopsis thaliana*. Located at the N-terminal of specific plant sHSPs is a sequence that provides the information that targets the sHSP to the correct cellular compartment; shown in olive green. Important structures such as alpha helices and beta sheets are also indicated. The first four sHSPs, HSP26.5-MII, HSP25.3, HSP23.5-MI/C, HSP23.6-MI/C, are the focus of this study. HSP26.5-MII localizes only to the mitochondrion. HSP25.3-P localizes only to the chloroplast. The other two sHSPs, HSP23.5-MI/C and HSP23.6-MI/C, localize to both the mitochondrion and the chloroplast under heat stress conditions.

Information on gene expression of *Arabidopsis* genes in specific plant tissues can be found on the ePlant database (https://bar.utoronto.ca/eplant/) . The data show that sHSPs are elevated in early development and localized in the seeds and in the root (Figure 4). There is also some expression in the reproductive structures of the *Arabidopsis* plants.

There is very little literature regarding the two sHSPs, HSP23.5-MI/C and HSP23.6-MI/C, besides that they localize in both the chloroplast and the mitochondria (Van Aken et al., 2009). The Vierling lab has already identified HSP23.5-MI/C and HSP23.6-MI/C single knockout mutant in Arabidopsis, as well as the higher order HSP23.5/23.6 double knockout. They show no noticeable difference in phenotype under normal growth conditions compared to wild-type plants, but further experimentation is required to observe phenotypes.

There is great challenge in trying to develop a mechanistic understanding of how sHSPs function in vivo. Determining how sHSPs affect plant survival may provide insight as to how they might function.

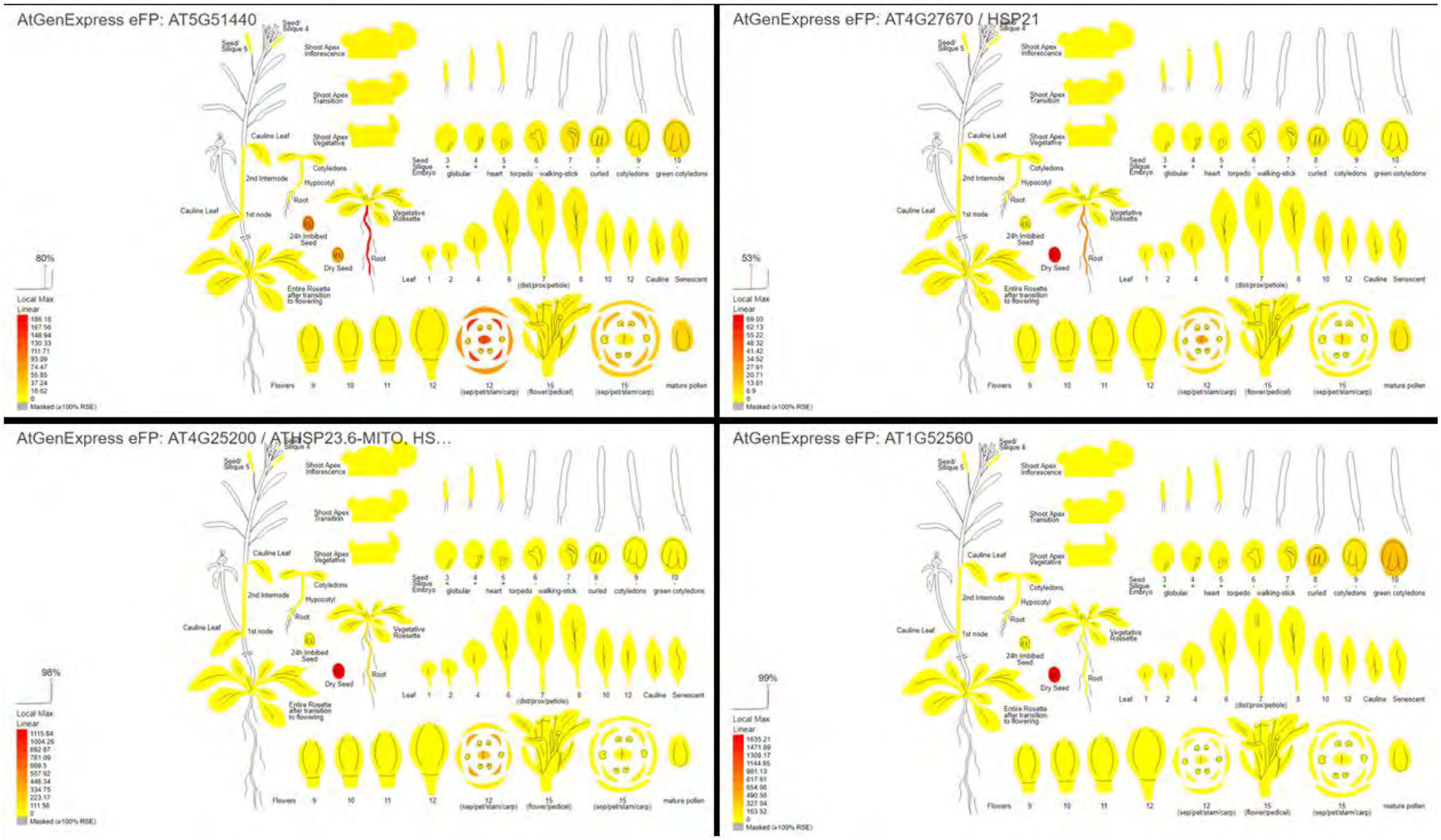

Figure 4. Gene expression of *Arabidopsis* sHSPs. The ePlant database information on expression levels for the sHSPs, *hsp23.5* (AT5G51440; top left), *hsp23.6* (AT4G25200; bottom left), *hsp25.3* (AT4G27670; top right), *hsp26.5* (AT1G52560; bottom right). The pictures are from the AtGenExpress eFP viewer. The data come from Schmid et al., 2005, Nature Genetics 37:501 and Nakabayashi et al., 2005, The Plant Journal, Vol 41:697. Gene expression data were generated by the Affymetrix ATH1 array and were normalized by the GCOS method, TGT value of 100.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* lines used in this study were of the Columbia (Col-0) background unless stated otherwise. Seeds were sterilized using a 50% bleach solution (50% bleach, 0.1% Triton-X100). All seeds were plated in a specific manner (specified below) in plates containing MS media (0.5X Murashige and Skoog basal medium powder, 0.8% agar, and a varying amount of sucrose [0%, 0.5%, 1.0%]). Seeds on plates were stratified at 4 ºC in darkness for 3 days to synchronize germination before moving them into a growth chamber at 22 ºC under long day (16 h light and 8 h dark) conditions, unless stated otherwise. Plants that were transplanted were carefully transferred from the MS media to pots with soil so that their roots were intact. Transplanted seedlings were kept in humid conditions in the growth chamber for 3 days.

Creating transformants via floral dipping (Conducted by Dr. Kim)

Agrobacterium tumefaciens carrying the genes of interest will be are selected for through kanamycin resistance. Once resistant colonies are grown in LB media (final concentration 10% peptone, 5% NaCl, 5% yeast extract) and then resuspended to a 5% sucrose solution to an OD_{600} of 0.8. Silwet L-77 was then added (final concentration 0.05% v/v). Plants with immature flower clusters were then dipped into the solution for 3 seconds with gentle agitation until a film of liquid coated the plant. Dipped plants were kept in humid conditions for 24 hours after which they were allowed to grow under normal conditions.

DNA Extraction

A small leaf from each F2 generation plant was harvested into a 1.7mL Eppendorf tube. A blue pestle was used to grind the plant tissue into a paste. The mixture was then incubated in 150 µL of DNA extraction buffer for 5 min and then another 5 min after adding isopropanol. After the two incubation steps, the sample was centrifuged for 5 min and the supernatant was discarded. 70% ethanol was added to the sample and centrifuged for 5 min before discarding the supernatant. After allowing the sample to dry for 40 min, the DNA was resuspended in 1X TE (10 mM Tris HCl, 1.0 mM EDTA, pH 8) buffer by vortexing and incubation at 4 ºC overnight. DNA was stored in the dark at -20 ºC after resuspension.

Before using the DNA for PCR reactions, the samples were centrifuged at maximum speed to remove unwanted plant material.

Genotyping for hsp23.5 (AT5G51440)

Polymerase chain reaction (PCR) was utilized to genotype for *hsp23.5*. Genomic DNA extracted from plants of interest was amplified in two ways, using primers to detect the wild-type gene or primers to detect the mutant allele carrying the T-DNA insertion. The wild-type primers were:

23.5-F2 5'- GCACGACGAGTTAACCCATC -3'

23.5-R2 5'- AAACCTCCGTCCATCTCCAG -3'.

The primers to genotype for the SALK_118536 T-DNA insertion were:

23.5-R2 5'- AAACCTCCGTCCATCTCCAG -3'

LBb1.3 5'- ATTTTGCCGATTTCGGAAC -3'.

The PCR mixture was a total of 20 µL (final concentration 1X Phusion HF Reaction Buffer from NEB, 0.5 µM forward primer, 0.5 µM reverse primer, 200 µM dNTP each, homemade Phusion

polymerase titrated for optimum concentration, and milli Q water). The PCR conditions were: 1 cycle at 95 ºC for 1 min for initial denaturation followed by 40 cycles at 95 ºC for 10 sec, 63 ºC for 20 sec, and 72 ºC for 30 sec for denaturation, annealing, and extension, respectively, and finishing with a 5 min incubation at 72 ºC and an infinite hold at 4 ºC. 6X DNA loading dye (final concentrations 5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol FF, and 0.04% (w/v) Orange G) was added to the reaction mixture. 10 µL of the reaction mixture with dye was loaded on a 1.3% agarose gel made with 1X LAB (10 mM lithium acetate, 10 mM boric acid) buffer and run at 250 V for 15 min. The gel was visualized with a G:Box iChemi XT(Syngene).

Genotyping for hsp23.6 (AT4G25200)

PCR was utilized to genotype for *hsp23.6* as described for *hsp23.5*, but with the following primers. The wild-type primers were:

23.6-F2 5'- AACAGGCCTAATACCGATGG -3'

23.6-R2 5'- CATCGACCGTGCCAAACTAC -3'.

The primers to genotype for SAIL_373_B09 T-DNA insertion were:

23.6-R2 5'- CATCGACCGTGCCAAACTAC -3'

SAIL-LB3 5'- TAGCATCTGAATTTCATAACCAATCTCGATACAC -3'.

Genotyping for hsp25.3 (AT4G27670)

Because the *hsp25.3* allele is a point mutation in the 3' splice site of the gene, a derived cleaved amplified polymorphic sequences (dCAPS) assay was used for genotyping. The wild-type sequence is shown below. In the mutant, there is a change from **G** to A right before the start of the second exon.

CAACGCTTAACCATGGACGTCTCTCCTTTCGGTACGTTCAACTAACTAAACGGACTT
CACTTGTATAAATAAAAACTACATCCTCTGCTTATTACTTAAAACATTGCTCTGTTTT
TATGAACTCAGCTTTTGTCAATCGAAAATCTCTTCCTCTGCTTTGAACTGAAACAATG
TTCTGTTTTAATCTAACCACA**G**GATTGTTGGATCCTTTGTCACCAATGAGGACGATG
CGACAAATGTTAGATACTATGGACAGGATGTTCGAGGACACTATGCCTGTCTCAGGA
AGAAACAGAGGAGGAAGTGGAGTGTCAGAGATTCGTGCACCGTGGGACATCAAAG
AGGAAGAACACGAGATCAAGATGCGTTTCGACATGCCTGGTCTCTCTAAAGAAGAC
GTCAAAATCTCTGT

The forward primer is cut in the mutant by BstXI (recognition sequence: CCANNNNNNTGG). Only the mutant is cut because the restriction enzyme which cuts at this highly specific site can only recognize the sequence in the mutant. The wild-type sequence does not contain the restriction site, and therefore the restriction enzyme cannot recognize the site. PCR was used to amplify the extracted genomic DNA. The primers used were:

25.3p-3 F 5'- AAACAATGTTCTGTTTTAATCTAACCACC -3'

25.3p-4 R 5'- AGAGACCAGGCATGTCGAAA -3'.

The PCR mixture was a total of 20 µL (with final concentration 1X Standard Taq Reaction Buffer, 0.5 µM forward primer, 0.5 µM reverse primer, 200 µM dNTP each, 0.1 µL homemade Taq polymerase, and milli Q water). The PCR conditions were initial denaturation at 95 ºC for 2 min followed by 40 cycles of 95 ºC for 30 sec, 57 ºC for 30 sec, and 72 ºC for 20 sec for denaturation, annealing, and extension respectively and then finishing with a 10 min incubation at 72 ºC and an infinite hold at 4 ºC.

The restriction enzyme digestion was performed in a 13 µL reaction (11.4 µL of PCR reaction, 1X NEB3.1 buffer, 0.3 µL BstXI restriction enzyme). The mixtures were incubated at

37 ºC for 4 h and then at 12 ºC for 30 min. 6X DNA loading dye was added to the reaction mixture. 10 µL of the reaction mixture with the dye was loaded on a 3.0% agarose gel made with TAE (40 mM Tris Base, 20 mM glacial acetic acid, 1 mM disodium EDTA) buffer and run at 100 V for 45 min. The gel was visualized as above.

Genotyping for hsp26.5 (AT1G52560)

PCR was utilized to genotype for *hsp26.5*. See *Genotyping for hsp23.5* protocol. The wild-type primers were:

26.5m-1 5'- TCTAGCTCGTCTGGCTTTGAG -3'

26.5m-2 5'- AAGAACACAAAAACGACACCG -3'.

The primers to genotype for SAIL_423_G06 T-DNA insertion were:

26.5m-1 5'- TCTAGCTCGTCTGGCTTTGAG -3'

SAIL LB3 5'- TAGCATCTGAATTTCATAACCAATCTCGATACAC -3'.

Protein extraction and quantification

Approximately 80-90 mg of whole seedlings were flash frozen and ground before adding three volumes of sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 65 mM DTT, 15% sucrose, 0.01% bromophenol blue). Mixtures were heated and centrifuged at maximum speed to isolate the supernatant containing total protein. 2 µL of BSA standards (sample buffer (blank standard), 0.125 mg/mL 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL BSA) and samples were spotted on filter paper and left to dry overnight. The filter paper containing the spots was incubated in Coomassie Stain (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) for 10 min then destained with deionized water for 30 min. Each spot was hole-punched into a tube containing 2% SDS and incubated at room temperature for 4 h with

agitation. The samples were then quantified using a Nanodrop Spectrophotometer by comparing absorbances to the BSA standards. The protein concentrations were between 1-2 ug/uL for most samples.

Immunoblot analysis

50 µg of total protein from Col-0, *hsp23.5,hsp23.6,* and *hsp26.5* extracted with sample buffer and denatured with heating at 95°C for 5 min was separated on 15% SDS-PAGE and blotted for 2 h to nitrocellulose membrane using semi-dry transfer. Blots were blocked with 5% (w/v) milk in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature with gentle agitation and rinsed with TBS-T. Blots were then incubated in primary antibody diluted to 1:2000 in TBS-T for 1 h at room temperature (RT) with agitation. The antibody solution was decanted, and the blot was rinsed briefly twice, then washed 3 times for 10 min in TBS-T at RT with agitation. Blot was incubated in GE Healthcare secondary antibody (anti-rabbit IgG horseradish peroxidase conjugated) diluted to 1:5000 in TBS-T for 1 h at room temperature with agitation. Blot was washed as above and incubated with Thermo Scientific SuperSignal West Femto Maximum Sensitivity ECL Substrate before visualizing with the G:Box iChemi XT (Syngene).

Hypocotyl elongation assay

The hypocotyl elongation assay was performed as described by Kim et al., 2017. The number of seeds needed for each genotype were sterilized (50% bleach and 0.1% Triton X-100) for 10 min. Seeds were plated on 100 x 15 mm square petri dishes that contained 10 mL plant media (0.5X Murashige and Skoog media, 0.5% sucrose, 0.8% agar) in a sterile environment. Seed placement was staggered on each line to avoid contact between seedlings during growth. The plates were wrapped in Parafilm and placed in 4 ºC for 3 days to synchronize germination.

Plates were wrapped with aluminum foil to ensure seedlings were kept in the dark. The plates were put in the growth chamber at 22 ºC for 3 days to germinate and grow vertically. The plates were then unwrapped and placed horizontally in an incubator in the dark set at 38 ºC for 1.5 h (acclimation treatment). A replicate of this plate was kept at 22 ºC (the room temperature control). The plates were taken out of the incubator and kept vertically at 22 ºC for 2 h in a dark place for the recovery period and then horizontally placed into a 45 ºC incubator for a variable amount of time (2.5 h, 3 h) for the heat treatment. After the heat treatment, plates were marked at the tip of each hypocotyl, wrapped in aluminum foil, and then placed vertically at 22 ºC for 3 days. The elongation of each hypocotyl was measured after the last recovery period.

Thermomemory heat stress assay

See above for plate preparation and stratification. The plates were unwrapped and placed horizontally in an incubator in the dark set at 38 ºC for 1.5 h (acclimation treatment). The plates were taken out of the incubator and kept vertically at 22 ºC for a variable number of days (2 d, 3 d) in a dark place for the recovery period and then horizontally placed into a 45 ºC incubator for 45 min for the heat treatment. After the heat treatment, plates were marked at the tip of each hypocotyl, wrapped in aluminum foil, and then placed vertically at 22 ºC for 3 days. The elongation of each hypocotyl was measured after the last recovery period.

Light grown seedlings heat stress assay

Modified light grown seedlings assay was performed as described by Kim et al., 2017. The number of seeds needed for each genotype were sterilized (50% bleach and 0.1% Triton X-100) for 10 min. Seeds were plated on circular petri dishes that contained 25 mL plant media

(0.5X Murashige and Skoog media, 0.5% sucrose, 0.8% agar) in a sterile environment. Seed placement was staggered on each line to avoid contact between seedlings during growth. The plates were wrapped in gas permeable tape (which allowed for gas exchange) and placed in 4 ºC for 3 days to synchronize germination. The plates were put in the growth chamber at 22 ºC for 10 days to germinate and then placed in an incubator in the dark set at 38 ºC for 1.5 h for acclimation treatment. A replicate of this plate was kept at 22 ºC (the room temperature control). The plates were taken out of the incubator and kept at 22 ºC for 2 h in the growth chamber for the recovery period and then horizontally placed into a 45 ºC incubator for a variable amount of time (3 h, 4 h, 5 h, 6 h) for the heat treatment. After the heat treatment, plates were left to recover in the growth chamber at 22 ºC for 7 days. Sensitivity to heat was indicated by the bleached white appearance of the seedlings.

RESULTS

Obtaining the chloroplast sHSP triple knockout mutant

Previous experiments revealed that transcript levels of *hsp23.5*, *hsp23.6*, *hsp25.3*, *hsp26.5* were highly elevated when plants are heat stressed. These proteins can accumulate to a significant proportion of the plant's total protein content, yet the function of these proteins is unknown. To determine the role of these proteins in thermotolerance, we first needed to create multiple gene knockouts of various sHSPs to test in different phenotypic assays. Single knockouts of these four genes were already available in the Vierling lab. A double knockout (DKO) of *hsp23.5* and *hsp23.6* had also been generated. After the DKO was obtained, a higher-order knockout mutant was also obtained; the mitochondrial sHSP triple knock out (mTKO) was generated (*hsp23.5*, *hsp23.6*, and *hsp26.5)*. The first task of this study was to genotype a cross that Dr. Kim had done to determine whether a chloroplast sHSP triple knockout (cTKO) was

viable. A complication to obtaining this mutant combination was that the *hsp23.6* and *hsp25.3* genes are located on the same chromosome, with the distance between these two genes being approximately 5 centimorgans. For this reason, 64 seedlings of the F1 generation from the parent plant with genotype *hsp23.5*, *hsp23.6*/+, and *hsp25.3*/+ were genotyped.

Of the 64 seedlings, 5 plants were confirmed to be cTKOs (Figure 5 and Figure 6). These five samples are identified as 141_98_6, 141_98_16, 141_98_19, 141_98_22, and 141_98_25. The knockout of *hsp23.5* and *hsp23.6* is the result of a T-DNA insertion in each gene, which does not allow the plant cell to express these protein chaperones. This is useful because with the correct DNA primers, it is possible to obtain T-DNA mutant or wild-type gene products using the polymerase chain reaction (PCR), followed by agarose gel visualization of the products. The T-DNA amplicon product, which identifies the mutant gene, is smaller than that produced from the wild-type gene. The logic is that when gene specific primers are used during the PCR, if the genomic DNA does not contain the T-DNA for the *hsp23.5* or *hsp23.6*, then the PCR products will be approximately 1000 bp in size; however, if the T-DNA insertion is present, then there would be no product. When the T-DNA insertion is present, there is no product made with the gene specific primers, although there is a product of approximately 700 bp with the T-DNA primers. After performing gel electrophoresis, the presence of no band at 1000 bp and a band indicating T-DNA would suggest that the gene is knocked out, and the knockout is homozygous. Since the DNA piece that is 1000 bp is longer than 700 bp, it will travel less distance in the gel. All of the five cTKO candidates showed no wild-type bands after the PCR reaction, but did show T-DNA bands for *hsp23.5* and *hsp23.6* (Figure 5).

The process for determining whether the plant is a knockout for *hsp25.3* is different, because instead of a T-DNA insertion the gene has a point mutation. For this specific mutation, a

method known as derived cleaved amplified polymorphic sequence (dCAPS) was used. This method uses a primer that is mismatched to the template DNA by one base pair, introducing a

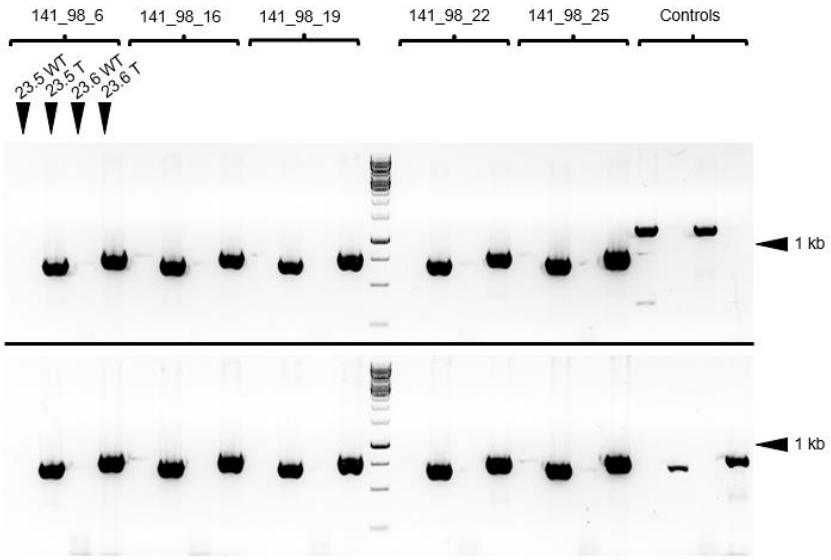

Figure 5. **Genotyping results for *hsp23.5* and *hsp23.6***. The five cTKO candidates shown (141_98_6, 141_98_16, 141_98_19, 141_98_22, and 141_98_25) that were determined to be knockouts for *hsp23.5* and *hsp23.6*. The control sample was Col-0 DNA. "23.5 WT" represents the PCR reaction using primers: 23.5-F2 5'- GCACGACGAGTTAACCCATC -3' and 23.5-R2 5'- AAACCTCCGTCCATCTCCAG -3'. "23.5 T" represents the PCR reaction using primers: 23.5-R2 5'- AAACCTCCGTCCATCTCCAG -3' and LBb1.3 5'- ATTTTGCCGATTTCGGAAC -3'. "23.6 WT" represents the PCR reaction using primers: 23.6-F2 5'- AACAGGCCTAATACCGATGG -3' and 23.6-R2 5'- CATCGACCGTGCCAAACTAC -3'. "23.6 T" represents the PCR reaction using primers: 23.6-R2 5'- CATCGACCGTGCCAAACTAC -3' and SAIL-LB3 5'- TAGCATCTGAATTTCATAACCAATCTCGATACAC -3'. The wildtype PCR product is expected to be slightly larger than 1 kb. The T-DNA PCR product is expected to be approximately 700 kb. The top half of the gel was loaded with a wild-type control to indicate that the primers are amplifying correctly. The bottom half of the gel used knockout controls from each of the two genotypes to indicate that those primers are amplifying correctly.

restriction site that can be recognized by a restriction enzyme, in this case *Bst*XI. After PCR amplifies the region containing the point mutation, the products are digested with *Bst*XI, and agarose gel electrophoresis is performed. If there is no mutation, then the digest would be

unsuccessful and result in a single band, but if there is a point mutation and the plant was heterozygous for this mutation, then there would be two bands (one shorter than the wild-type since the DNA fragment was cut) indicating that the restriction enzyme was able to recognize the restriction site. If there is only one lower band, this indicates the sample is a homozygous knockout of *hsp25.3*, as is the case with the five samples (Figure 6).

Seeds from the mature cTKOs were harvested. The cTKO genotype described in this study will be from the 141_98_6 line, chosen because the genotyping data were clearest from this sample. Under optimal growth conditions in a growth chamber these plants have no obvious phenotypes that distinguishes them from wild-type Col-0.

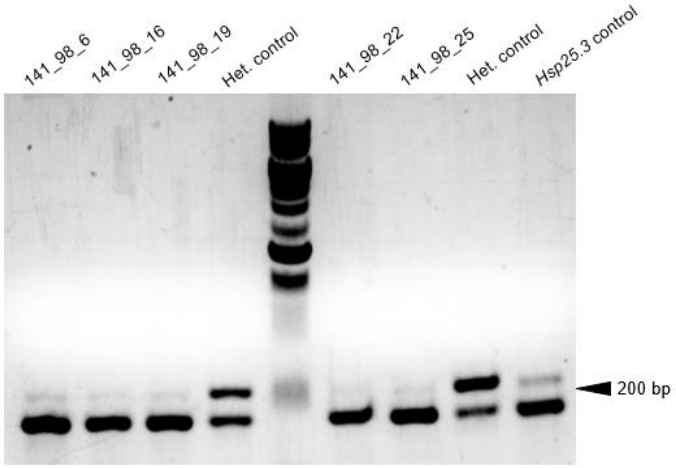

Figure 6. **Genotyping results for *hsp25.3*.** The five candidates shown (141_98_6, 141_98_16, 141_98_19, 141_98_22, and 141_98_25) that were determined to be knockouts for *hsp25.3*. The PCR product is 222 bp when the template DNA is amplified with primers 25.3p-3 F 5'-AAACAATGTTCTGTTTTAATCTAACCACC -3' and 25.3p-4 R 5'-AGAGACCAGGCATGTCGAAA -3'. When the PCR product is digested by the *BstXI* restriction enzyme, wild-type samples are not cut and remain 222 bp (upper band) and mutants produce 190 bp (lower band) and 32 bp (not present on the gel) fragments. To make sure that the *BstXI* restriction enzyme digest proceeded to completion, DNA from a *hsp25.3* plant was tested as a control.

Immunoblotting for sHSPs

To confirm that knockout plants are not producing any of the sHSPs, immunoblotting was performed. We first needed to confirm the specificity of the antibodies. For this purpose, it was more useful to use the single mutant knockouts *hsp23.5*, *hsp23.6*, and *hsp25.3* (the antibody for *hsp25.3* had already been obtained). The antibodies against these three proteins were generated from rabbits inoculated with the protein (Hsp25.3) or a protein-specific peptide (Hsp23.5 and Hsp23.6) by Agrisera, a Swedish antibody company. The antibody tested against HSP23.5-MI/C seemed to be non-specific for the protein, as no band was observed between 17 and 28 kDa (Figure 7).

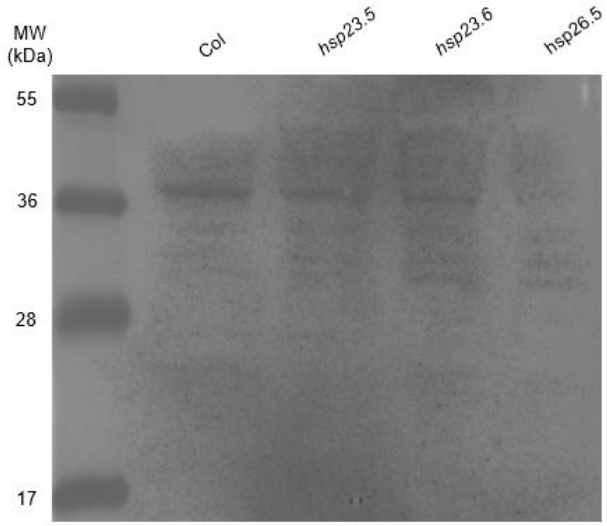

Figure 7. Western blot analysis for HSP23.5. Total protein from different genotypes of *Arabidopsis* was extracted, separated by SDS-PAGE, and blotted onto nitrocellulose.

The antibody against HSP23.6-MI/C seemed to be binding to the expected protein because of the observed band at approximately 24 kDa. Further, Col-0, *hsp23.5* and *hsp26.5* all express

HSP23.6-MI/C and the antibody detects this protein in these genotypes, but does not detect it in *hsp23.6* proteins as anticipated, because this genotype is null for HSP23.6-MI/C (Figure 8).

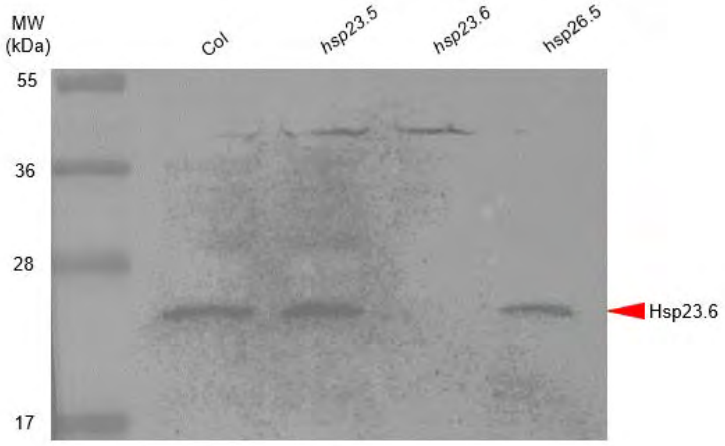

Figure 8. Western blot analysis for HSP23.6. Total protein from heat-stressed A*rabidopsis* tissue was extracted, separated through SDS-PAGE, and blotted onto nitrocellulose.

The antibody against HSP26.5-MII detects a band of the expected size of approximately between 17 kDa and 28 kDa for all the genotypes that express this protein and do not see a band in *hsp26.5* extracts (Figure 9). There seems to be nonspecific antibody binding to a protein of similar size that is present even when the plants are not heat stressed (Figure 5). Although the identity of this other protein is unknown, we conclude that this antibody recognizes HSP26.5-MII.

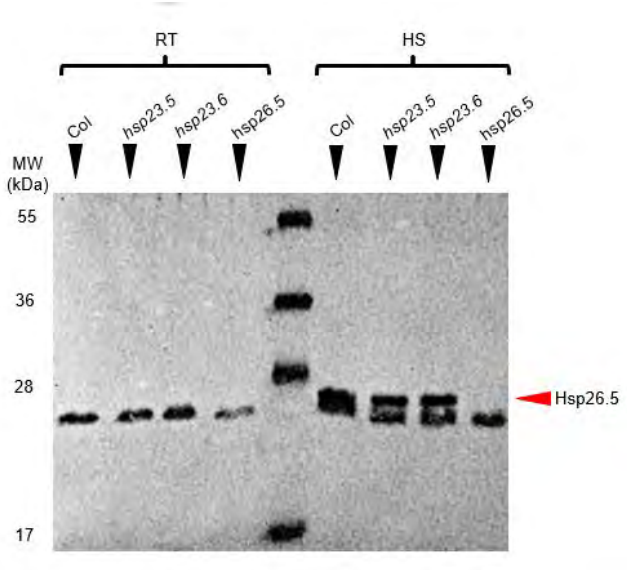

Figure 9. Western blot analysis for HSP26.5. Total protein from heat stressed *Arabidopsis* tissue was extracted, separated through SDS-PAGE, and blotted onto nitrocellulose.

Stress Assays

Under normal growth conditions, the phenotype of the different mutants is not obviously different than the wild-type. Plants start to express a high level of sHSPs after being exposed to temperatures of approximately 37-38 ºC. As higher-order sHSP knockouts are obtained, the logical next step is to heat stress plants of different genotypes and compare them to determine whether the absence of sHSPs leads to decreased stress tolerance. Different stress assays will be utilized to determine the phenotypes of sHSP knockouts. The hypocotyl elongation assay to assess hypocotyl growth after heat stress conditions, developed by Dr. Kim et al. (2017), is one such assay to gauge thermotolerance used to see the effects of acute heat stress on plant growth of sHSP mutants in comparison to the wild-type. The hypocotyl elongation assay and heat stress assay for light-grown 7-10 day old seedlings was modified and utilized for various heat stress treatments.

Hypocotyl Elongation Assay

It is commonly accepted that plants are able to acclimate to temperatures that are above the optimal temperature for growth. Typically, acclimation requires a period of exposure to a non-damaging temperature treatment, above the optimal temperature for growth (Kim et al., 2017). It takes plants a few hours of recovery after the heat acclimation treatment to be able to tolerate normally lethal temperatures. Dr. Kim extended this simple hypocotyl elongation assay designed to investigate the relationship of heat acclimation to the heat shock response done in soybeans to *Arabidopsis* and can be used to identify mutants with altered thermotolerance.

After 2.5 and 3 hours of heat stress, most of the genotypes are affected by heat stress (Figure 10). The DKO, 26.5m, and cTKO genotypes are comparably sensitive to heat treatment, and more sensitive than wild type. The mTKO is the most sensitive genotypes, since the growth of the hypocotyl is reduced by the greatest amount (>20% compared to wild-type). This result makes sense because the mTKO is a combination of the DKO and 26.5m genotypes, and the knockout of these genes has an additive effect. The phenotype of the cTKO is most likely due to same genes that are knocked out in the DKO since the cTKO is a combination of the DKO and 25.3p genotypes. It is unclear which gene in the DKO contributes to the reduced heat tolerance.

These data indicate that there is a heat-sensitive phenotype associated with the *hsp23.5/hsp23.6* and *hsp26.5* genes, but no apparent heat-sensitive phenotype of *hsp25.3* for seedlings grown and heat stressed in the dark as described. The heat sensitivity is significantly less than that of a mutant in the chaperone Hsp101, the *hot1-3* mutant, which was used as a heat-sensitive control in these experiments (Hong and Vierling, 2001).

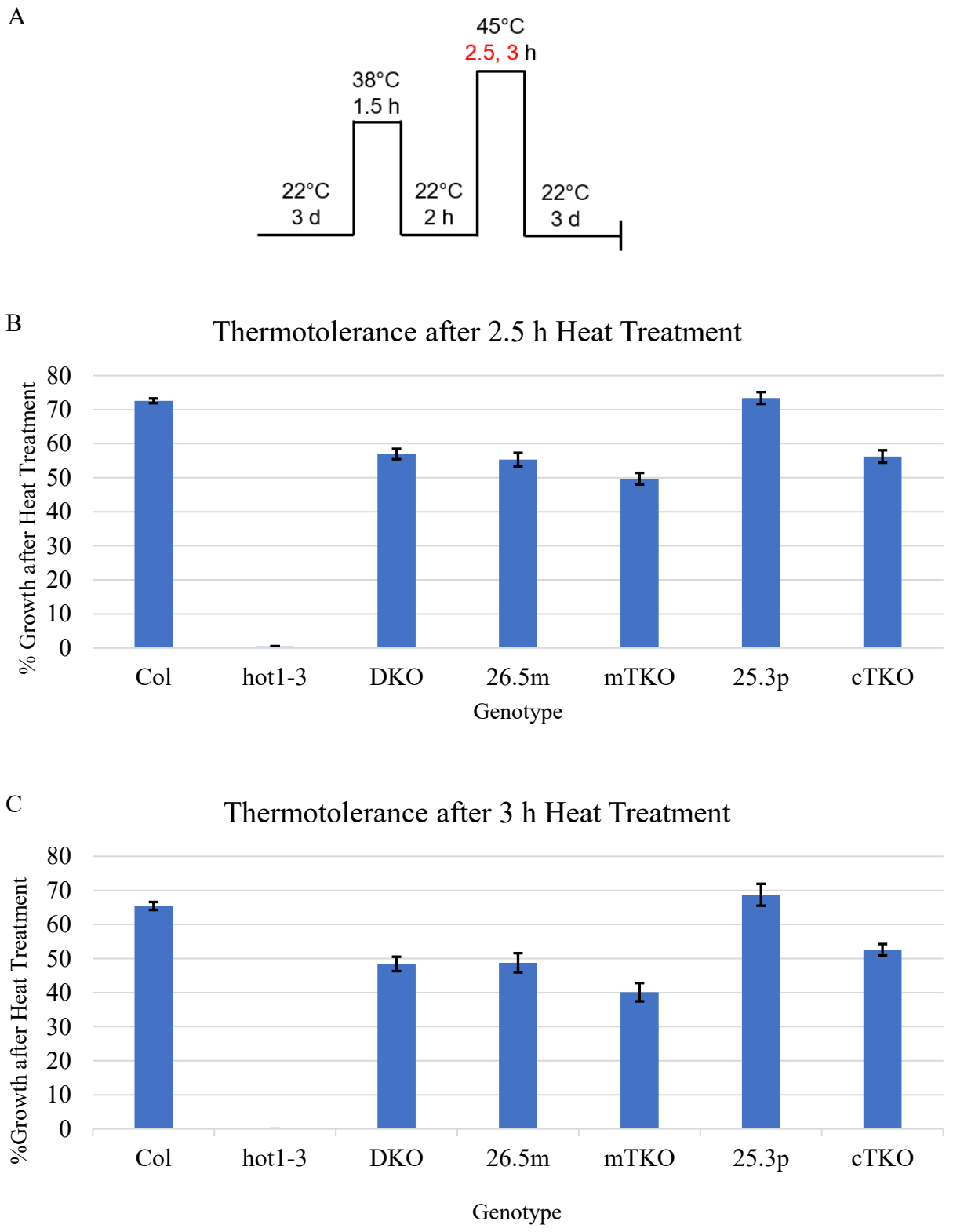

Figure 10. Hypocotyl elongation assay comparing different genotypes. A.) Schematic of the protocol used for heat stress. Plants were grown in the dark at 22 ºC for 3 days, acclimation

treated at 38 ºC for 1.5 h, allowed to recover at 22 ºC for 2 h, then subjected to heat stress at 45 ºC for either 2.5 h or 3 h before measuring the hypocotyl growth 3 days later. B.) Results of the 2.5 h heat treatment. C.) Results of the 3 h heat treatment. Note: 25.3p refers to *hsp25.3* and 26.5m refers to *hsp26.5*. *hot1-3* is a null mutant of the Hsp101 chaperone. The assay was repeated three times with approximately 16 seedlings per replicate. The error bars represent standard error.

Hypocotyl Elongation Assay for Thermomemory

Thermomemory and other stress memory have been largely unexplored in plants. Sedaghatmehr and colleagues (2016) did a study to determine the effects of HSP25.3 and FtsH6, a plastid metalloprotease, on thermotolerance in *Arabidopsis*. The researchers hypothesize that priming and stress memory might involve metabolic changes that are maintained throughout the memory phase, thus allowing a more rapid response of the plant to an upcoming new stress. To confirm the importance of HSP25.3 to thermomemory, we modified the hypocotyl elongation assay to measure the effects of the absence of sHSPs.

The results obtained were different than previously reported. Sedaghatmehr et al. (2016) observed that a HSP25.3 amiRNA line showed reduced growth compared to wild-type in a thermomemory test. In contrast, our experiment showed the opposite; the mutants grew somewhat better than wild-type plants when a long recovery period preceded the severe heat stress (Figure 11). All of the sHSP mutants grew as well as, or better than the wild-type Col-0.

Heat Stress Assays of Light Grown Seedlings

sHSPs have been proposed to interact with the photosystems in chloroplasts that absorb light (Neta-Sharir et al., 2005). To investigate the effects of the sHSP knockouts in the presence of light, the light grown seedling assay was modified (Kim et al., 2017). The ideal parameters for this experiment were not previously determined. Therefore, a number of different conditions

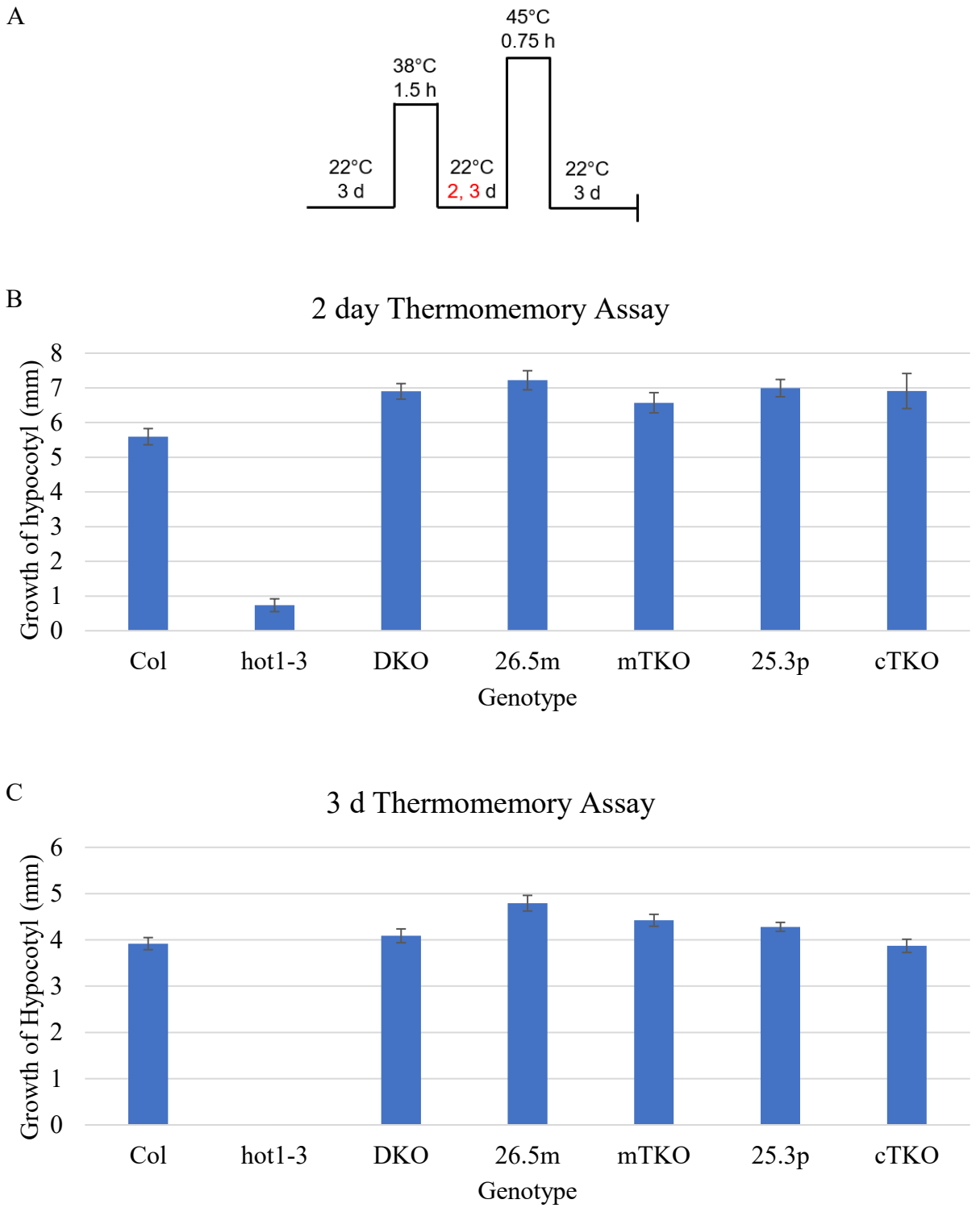

Figure 11. Hypocotyl elongation assay for thermomemory comparing different genotypes. A.) Schematic of the protocol used for heat stress. Plants were grown in the dark at 22 ºC for 3 days, acclimation treated at 38 ºC for 1.5 h, allowed to recover at 22 ºC for either 2 or 3 d, then subjected to heat stress at 45 ºC for 0.75 h, and hypocotyl growth was measured 3 days later. B.)

Results of the 2 d recovery period. C.) Results of the 3 d recovery period. Note: 25.3p refers to *hsp25.3* and 26.5m refers to *hsp26.5*. The assay was repeated three times with approximately 16 seedlings per replicate. The error bars represent standard error.

were tried such as varying the sucrose concentration (using either 0%, 0.5% or 1.0% sucrose), using different volumes of MS media (10 mL or 25 mL), and using gas permeable tape that allows for gas exchange rather than parafilm.

The best conditions to grow the seedlings in the light was determined to be with 25 mL of plant media containing 0.5% sucrose and sealing the plates with gas permeable tape to allow for gas exchange. However, plants heat stressed in the light resulted in too much variability (Figure 12). We thought that heat stress in the light would be more severe than heat stress in the dark, because the plants are subject to a high temperature and light which could lead in some oxidative stress. However, to reduce variability, it is recommended that future stress treatments be done in the dark.

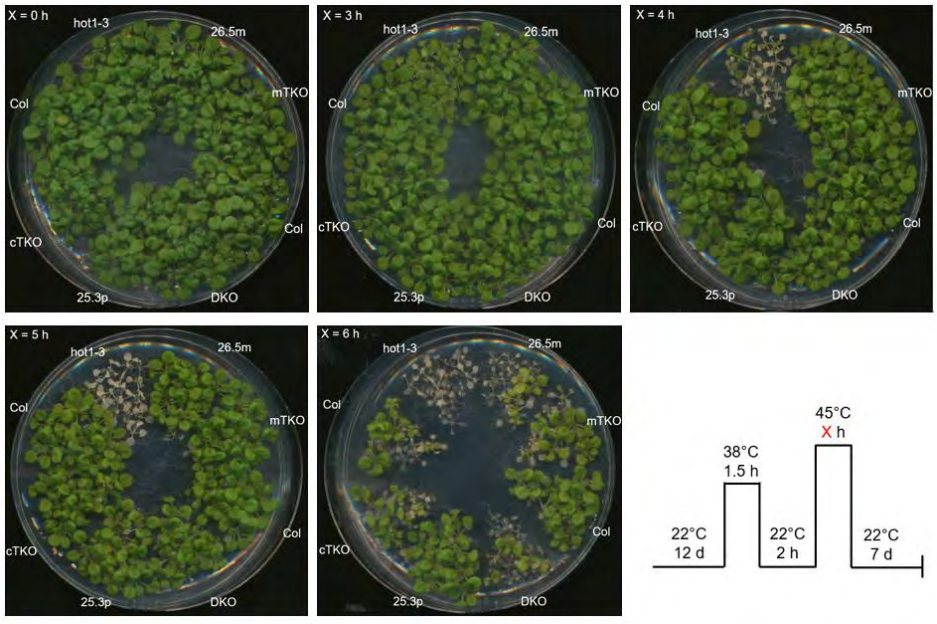

Figure 12. Light grown seedlings heat stress assay comparing different genotypes. The plants were grown in the light at 22 ºC for 12 days, acclimation treated at 38 ºC for 1.5 h in the light, allowed to recover at 22 ºC for 2 h, then subject to heat stress at 45 ºC for a variable numbers of hours X (red), before taking pictures 7 days later.

DISCUSSION

sHSPs are thought to help protect a variety of cellular functions that contribute to increased stress tolerance. We expected that sHSP knockout mutants would show reduced tolerance to heat, especially mutants that have multiple sHSP genes knocked out. In this study, I show that the genes for chloroplast localized sHSPs have been successfully knocked out and this genotype is viable. Immunoblotting confirmed that the mutant *Arabidopsis* plants do not express the corresponding sHSPs. Antibodies for HSP23.6 and HSP26.5 showed selectivity and the antibody for HSP25.3 had been previously obtained. The antibody for HSP23.5 did not show any apparent specific reactivity. To have a complete set of antibodies to detect each of these proteins, the next step is to obtain HSP23.5 antisera, which will be tried using a different peptide as antigen than was used previously. Pre-immune rabbit sera has been screened to identify appropriate animals to inoculate to generate an antibody against HSP23.5. Once a reliable antibody against HSP23.5 has been obtained, it will be possible to confirm the absence of sHSP expression in the knockout mutants, and also to determine the relative levels of these different proteins in the plant cell.

Obtaining the cTKO makes it possible to determine whether a sHSP quadruple knockout (QKO) mutant is viable by crossing the cTKO and the mTKO and genotyping the F2 generation. It should be viable because sHSPs are not abundantly expressed during normal growth conditions. However, if it is not viable, this might suggest that the sHSPs play a role in some developmental process because data from the ePlant database suggests that there is significant sHSP expression in seeds (Figure 4). The QKO mutant will have no sHSPs localizing in the mitochondria or the chloroplasts, which are the main sites for energy production in the plant. Currently, the cTKO and mTKO plants have been crossed and the F2 generation was screened.

Out of 96 plants, there appear to be two candidates that might be QKO mutants. I need to confirm the genotype through DNA sequencing, as the enzyme digestion for *hsp25.3* did not provide unequivocal data. These two candidates did not show any phenotype when grown under normal conditions.

The results of the hypocotyl elongation assay confirm that there is a heat-sensitive phenotype of the DKO, 26.5m, and mTKO. The phenotypes observed are associated with sHSPs localizing to the mitochondria, and the absence of these genes increases seedling sensitivity to heat. Although none of the knockout mutants were as sensitive as *hot1-3*, the DKO and 26.5m genotypes both showed more sensitivity to heat than wild-type; the percent growth of the hypocotyl for both of these genotypes was >10% lower than wild-type. The mTKO genotype was even more sensitive to heat since the percent growth was >20% lower than wild-type. Combining knock outs of multiple sHSPs seems to have an additive effect for hypocotyl growth. We need to conduct assays on the single knockout hsp23.5 and hsp23.6 mutants to determine which sHSPs account for the observed phenotype.

The chloroplast-localized sHSP triple knockout showed no observable phenotype in the assays employed, even when heat treated in the light. No conclusion could be drawn from these assays because results from the heat treatment was highly variable and not effective. It appeared that certain parts of the plates received either more light or more severe heat treatment than other parts of the same plate. After manipulating multiple variables in the light grown seedlings assay, I found the best parameters to reduce the variability in the plate assays. Future assays to assess the effects of the sHSP knockouts will have seedlings grown in the light, heat treated in the dark, and allowed to recover in the light. I expected the chloroplast-localized sHSPs knockout mutants to be more affected by the heat stress in the light than heat stress in the dark. My reasoning was

that during heat stress, chloroplast proteins would have no sHSPs to deal with misfolding, and subsequent exposure to light might jeopardize the plant because it cannot properly photosynthesize. However, this did not seem to be the case.

Although the light grown seedlings assay was not interpretable, an important observation was that the DKO and cTKO genotypes seemed to germinate later than the other genotypes, including wild-type. This phenotype might be connected to sHSP expression in the seed. It will be of interest to perform germination assays, as well as assays that involve heat stressing seeds to determine if there is any phenotype even before the development of the hypocotyl.

Exploring other types of stress might be of interest as well. Heat stress is not the only type of stress that causes expression of sHSPs. Plants are subject to other abiotic stresses such as oxidative stress, drought, and high soil salinity that can lead to protein misfolding and aggregation. It is possible that chloroplast-localized sHSPs are involved in managing protein misfolding in stresses other than heat stress.

In addition to determining the phenotype for the sHSPs, future experiments will also involve checking the expression of the sHSPs and other HSPs in sHSP knockouts through western blot analysis to gain insight to sHSP regulation. The amount of each sHSP expressed will be of interest to see whether sHSP levels in the mutants are different from wild-type. It is possible to check this by using purified sHSPs that serve as standards for total protein samples from *Arabidopsis*. Information about expression can lead to important insights to sHSP regulation, because establishing amounts of protein present after heat stress might help explain why plants show no phenotype in future assays; plant cells may compensate for the absence of certain sHSPs by upregulating genes for other sHSPs and HSPs. To find evidence for this hypothesis, expression levels must be determined.

Gaining more information about sHSPs and understanding the mechanism behind how these proteins work is critical. These proteins are thought to accumulate to significant levels in *Arabidopsis* when the plants are under heat stress and a lot can be learned the role of a protein by knocking it out, especially if shows an easily detectable phenotype. Knowledge of sHSPs has important applications, including being able to create more stress-resistant plants. Plants are not the only organisms that express sHSPs. Knowledge of sHSPs can also be applied to human protein-misfolding diseases and may possibly offer a different approach for treatment.

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