THESIS MANUSCRIPT

Using CRISPR Cas9 Mutagenesis to Understand the Function of Cytosolic Small Heat Shock Proteins in *Arabidopsis thaliana*

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Using CRISPR Cas9 Mutagenesis to Understand the Function of Cytosolic Small Heat Shock Proteins in Plants

Abstract

Heat shock proteins (HSPs) are molecular chaperones that are expressed in response to various types of stressors, however, they are upregulated most highly in heat stress conditions. Plants especially require chaperones because plants are sessile and, therefore, vulnerable to stressful environments. Small HSPs (sHSPs) are a conserved family of HSPs that prevent irreversible protein aggregation by binding to hydrophobic residues that are exposed on denaturing protein substrates; they act as "holdases" until other molecular chaperones refold or degrade the bound substrates.

In plants, sHSPs are divided into classes based on sequence identity and subcellular localization. Two classes of cytosolic sHSPs, class I (CI) and class II (CII) are highly expressed in response to heat in *Arabidopsis thaliana*. CI sHSPs are encoded by six genes: At3g46230 (HSP17.4), At1g59860 (HSP17.6A-I), At2g29500 (HSP17.6B-I), At1g53540 (HSP17.6C-I), At1g07400 (HSP17.8-I) and At5g59720 (HSP18.1-I), and CII sHSPs are encoded by two genes: At5g12020 (HSP17.6-II) and At5g12030 (HSP17.7-II). While it is possible that these two classes serve some redundant functions, recent evidence indicates that they likely have unique roles as well.

Since multiple sHSP genes of the same class can compensate for each other, studying the phenotypic effects of knockouts has been limited. However, using the new genome editing capabilities of CRISPR-Cas9, plants carrying a T-DNA insertion mutation of HSP16.7-CII were used to mutate HSP17.7-CII with CRISPR and five independent lines of CII sHSP double-knockouts (HSP17.6-II and HSP17.7-II) have been identified: three that carry deletions for the HSP17.7-II gene and two that have point mutations in the HSP17.7-II gene. The three CII sHSP double-knockout lines with the deletions in HSP17.7-II have also been confirmed to be null mutants by immunoblotting. Although the mutants show no phenotype under regular growth conditions, preliminary data suggest that there may be defects in thermotolerance.

In order to study the function of the CI genes, a vector containing four guide-RNAs that target each of CI genes at two sites was designed, generated and transformed into *A. thaliana*. Screening to identify plants from this transformation is in progress. Ultimately, identifying plants that are null for all six CI genes will allow definitive studies of their importance in heat stress tolerance.

This study is a first step in analyzing how the absence of entire classes of cytosolic sHSPs affects growth and stress tolerance in plants.

1: Introduction

Chaperones and the Heat Shock Protein Family

Proteins are biological macromolecules that are essential for carrying out cellular processes. Differences in the three-dimensional structure and folding of proteins allows them to perform a diverse set of functions that are wholly dependent on the specificity of this structure. Proteins adopt these conformations in an aqueous cellular environment to minimize solvent exposure of hydrophobic residues. Their conformations are then further directed by electrostatic interactions. Electrostatic interactions can also be strengthened or weakened, depending on local environmental conditions, increasing the diversity of possible conformations adapted by a protein. Although this flexibility in structure allows for proper protein function under specific cellular conditions, protein structure and function can be significantly disturbed by changes in cellular conditions.

Perturbing optimal cellular conditions can be stressful because as the normal structure of a protein changes, protein function can change or be lost. Stressors that can influence the structure and function of a protein include changes in temperature, pH, and salt concentration, among others. However, it is impossible for cells to completely avoid these stressors. Therefore, evolutionary pressure has been selected for specific proteins called molecular chaperones to manage "proteotoxic" stress. Molecular chaperones are diverse molecules that are able to refold proteins that are misfolded, as well as assist in folding proteins that are actively being translated (Mayer & Bukau, 2005; Rosenzweig et al, 2019).

Of the molecular chaperones, the heat shock protein (HSP) family is one of the most well-characterized. Although HSPs are upregulated in other conditions in addition to heat stress, they are termed "heat shock" proteins due to their exceptionally enhanced expression in high temperatures. HSPs can be classified as ATP-dependent or ATP-independent molecular chaperones. ATP-dependent chaperones, for example HSP70, use ATP to aid the refolding of unfolded proteins (Xu, 2018). However, these ATP-dependent chaperones do not function alone; they are a part of a network of chaperones that together, recognize and refold substrates. For example, protection and refolding of protein by the ATP-dependent chaperones can be facilitated by substrate interactions with small HSPs (sHSPs).

First discovered in *Drosophila melanogaster*, sHSPs are a conserved family of chaperones that are present in many other organisms including humans. They are likely essential to plants due to plant immobility and inability to escape high stress environments. Hence, it is important to determine how sHSPs interact and function in the stress response in plants. Ten separate families of sHSPs have been found in plants. Of these families, six are documented to be targeted to specific organelles, including the chloroplast, mitochondrion, ER, and peroxisome (Waters & Vierling, 2020). The four other families are thought to be cytosolic and are sometimes seen localized to specific granules within the cytosol. These "stress granules" are formed through liquid-liquid phase separation as a result of some external stressor. Composed of mainly proteins and nucleic acids, stress granules play a critical role in mediating the stress response and recovery pathway (Hofmann et al., 2021). Some of these cytosolic sHSPs are also thought to be able to move between the cytosol and the nucleus (Basha et al, 2010). The focus of the work presented here is on two families of cytosolic sHSPs in *A. thaliana*.

Significance of sHSPs

HSPs are an important class of proteins with implications ranging from as far as modern environmental problems to medical therapies for human diseases. Temperatures have been recently rising at an unprecedented rate due to climate change. This has many widespread societal effects, including reduced crop yields due to increasing temperatures. Hence, it is highly relevant to study proteins like HSPs, which play a pivotal role in conferring thermotolerance to plants. In humans, when certain sHSPs are mutated, it can lead to myopathies and retinopathies. Additionally, sHSPs, or the lack thereof, are thought to play a role in the progression of neurodegenerative diseases. sHSPs in humans have also been reported to be involved in cell growth and proliferation as well as the development of certain cancers (Xiong et al., 2020). Interestingly, both the upregulation and downregulation of sHSPs can lead to different types of cancer. For example, HspB1 overexpression promotes the progression of breast cancer cells in vitro and HspB8 underexpression inhibits the progression of breast cancer cells in vitro (Xiong et al, 2020). It has been proposed that sHSPs regulate apoptotic pathways by interacting with other signaling and protein quality control pathways. Since cancer cells also rapidly increase metabolic rates, sHSP overexpression in these cancer cells also protects these cells from apoptosis caused by oxidative stress through sHSPs interaction with the antioxidant defense network. There are even specific drugs that target HspB1, a cytosolic sHSP, that have been found to have anti-cancer therapeutic properties (Xiong et al., 2020). Mutations in some human sHSPs can also reduce their ability to prevent protein aggregation, which can give rise to neurodegenerative diseases like Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Overexpression of HspB1 has also been found to reduce aggregation and reduce Alzheimer's symptoms in mice models, while a lack of expression has been found to do the opposite (Vendredy et al, 2020). Similarly, in Parkinson's and Huntington's disease, sHSPs have been found to be associated with the accumulation of insoluble aggregates (Vendredy et al, 2020). Overall, sHSPs impact many different facets of plant and human biology.

sHSPs and a Proposed Model for their Function

sHSPs are ATP-independent molecular chaperones. At standard conditions, sHSPs are virtually undetectable in many plant tissues. However, they are expressed at high levels in plants during periods of heat stress, accounting for almost 1% of the cellular proteome. During heat stress, proteins are prone to denaturation. To minimize the interaction of the solvent with previously buried hydrophobic residues, denatured proteins can form insoluble aggregates that can interfere with other cellular processes and cause cellular damage. sHSPs competitively bind to these denaturing proteins before they are able to form aggregates to reduce proteotoxic stress. These sHSP-substrate complexes can then go on to interact with ATP-dependent chaperones like HSP70 or HSP101 that refold the damaged proteins (Mogk et al., 2003b).

sHSP monomers are between 12-42 kDa, but the majority assemble into multi-subunit oligomers of 12 or more subunits with a dimeric substructure. They consist of an alpha-crystallin domain with a variable N-terminal arm and a short C-terminal tail. The alpha-crystallin domain is a conserved domain among sHSPs that provides thermodynamic stability. The N-terminal arm of sHSPs is the most diverse in terms of amino acid sequence. Although the flexible C-terminus is not as conserved as the alpha-crystallin domain, it is not as diverse as the N-terminal arm. The C-

terminal arm has an I/L-X-I/L motif which is significant for oligomerization. However, this C-terminal motif alone is not sufficient to establish oligomerization. This is a common theme among sHSPs. Both oligomerization and substrate binding are not dictated by a single motif in the protein, which further complicates the study of these sHSPs (Basha et al., 2012).

HSP expression is mediated by heat shock factors (HSFs). However, HSFs regulate not only heat stress pathways, but they also coordinate multiple stress response pathways. HSFs are transcription factors that are induced by some type of cellular stress such as heat or drought. These transcription factors are key regulators for the complex stress response pathways in plants. In *A. thaliana*, HSFA2 expression increases from virtually undetectable to strongly detectable after heat stress (Guo et al., 2016). HSFs are also thought to be involved with multiple cell cycle pathways that regulate cell differentiation, development, and proliferation. Although it is known that HSFs are key transcription factors for stress-related genes such as HSPs, the exact mechanism by which they are activated and induce expression of downstream genes remains to be completely understood. Regardless, these HSFs are important regulatory molecules that facilitate the rapid accumulation of sHSPs in plants.

The exact mechanism of sHSP function remains substantially unknown. The current model proposes that sHSPs are “the first line of defense” for a cell. However, the relatively low expression of sHSPs prior to stress indicates that the model may be incomplete. Although there are tissues with constitutive expression in mammals, in plants, sHSPs only exist in low concentrations as oligomers at optimal growth temperature conditions. However, these oligomers do not actively prevent the aggregation of heat-sensitive proteins. The sHSP oligomers are proposed to be “reservoirs” for sHSP dimers, with the oligomers and dimers existing in dynamic equilibrium. This equilibrium highly favors the oligomeric species at standard conditions, but favors the dimeric species at heat stress conditions (Santhanagopalan et al. 2015). The dimeric sHSP is proposed to actively bind substrates at primarily hydrophobic residues, competing with the binding of other denatured substrates to prevent aggregation. Although, in the oligomeric state sHSPs do not actively bind substrates, the oligomeric structure is thought to be necessary to form larger sHSP-substrate complexes that coincide with sHSP function. As temperature increases, the expression of sHSPs increases and the equilibrium between oligomers and dimers shifts to favor the dimeric form. Elevated temperatures do not cause such a dramatic shift to dimers for some sHSPs. However, the oligomers still rapidly exchange subunits, which increases with temperature, and this increase in subunit-exchange facilitates chaperone activity; dissociation of dimers from oligomers exposes hydrophobic residues before they reassociate (Basha et al, 2010). Together, this drastically increases the availability of sHSPs and their capacity to bind substrates.

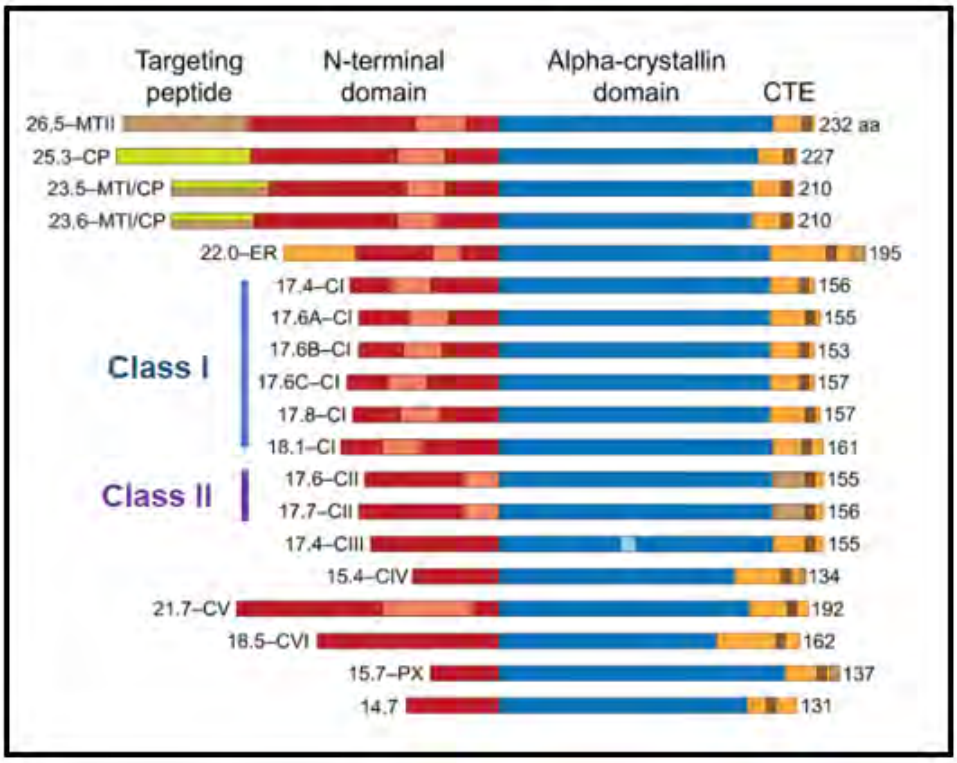

Figure 1. Different Classes of *Arabidopsis thaliana* sHSPs
The class I sHSPs consist of six genes: At3g46230 (HSP17.4-I), At1g59860 (HSP17.6A-I), At2g29500 (HSP17.6B-I), At1g53540 (HSP17.6C-I), At1g07400 (HSP17.8-I) and At5g59720 (HSP18.1-I). The class II sHSPs consist of two genes: At5g12020 (HSP17.6-II) and At5g12030 (HSP17.7-II). Both classes share a highly conserved alpha-crystallin domain shown in blue and the V/I X V/I motif in the C-terminus (brown box). However, the N-terminal domain (red) and the C-terminal extension (orange) have slight differences. Light orange regions in the N-terminus indicate conserved motifs unique to each class. Class I sHSPs share 70% protein sequence identity between any sHSP of the same class. Class II sHSPs share an 84% protein sequence identity between any sHSP of the same class. Yet, there is only a 30% protein sequence identity between any protein of these two different classes. Adapted from Waters & Vierling (2020).

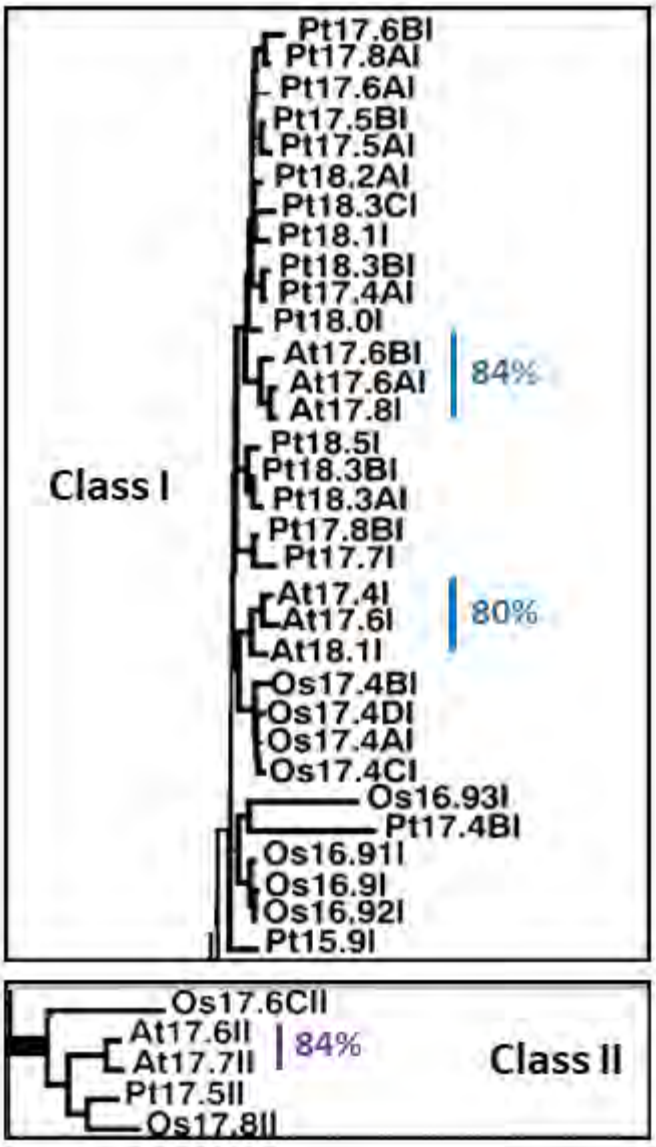

Figure 2. Phylogenetic Tree of Representative Angiosperm sHSPs
The three different species shown in the diagram are *Oryza sativa* (Asian rice), *Populus trichocarpa* (black cottonwood), and *A. thaliana*. TOP: The phylogenetic tree reveals that *A. thaliana* CI sHSPs can be categorized into two distinct clades. The first clade, consisting of At17.B-I, At17.6A-I and At17.8-I, have a protein sequence identity of 84%, while the second clade, consisting of At17.4-I, At17.6-I, and At18.1-I, has an 80% protein sequence identity. BOTTOM: The CII sHSPs are gene duplications found in a tandem array, which is also supported by the close evolutionary relationship. Adapted from Waters et. al, (2008).

Class I and Class II Cytosolic sHSPs in Plants

Plants have uniquely evolved distinct sHSPs targeted to different organelles and locations within the cell, which is not the case for other organisms. In plants, organellar and cytosolic sHSPs have been classified into different groups (**Figure 1**). Decades of research elucidated two main clades of cytosolic sHSPs, the class I (CI) and the class II (CII) sHSPs. In *A. thaliana* CI sHSPs consist of six genes: At3g46230 (HSP17.4-I), At1g59860 (HSP17.6A-I), At2g29500 (HSP17.6B-I), At1g53540 (HSP17.6C-I), At1g07400 (HSP17.8-I) and At5g59720 (HSP18.1-I), and CII sHSPs consist of two genes: At5g12020 (HSP17.6-II) and At5g12030 (HSP17.7-II). The CII genes are located only 1000 bp apart on chromosome 5 and are thought to be the result of a gene duplication event. Conversely, the six CI genes are scattered among different chromosomes within the genome, but they are also likely the result of gene duplication events. The sequence identity between the two CII genes is approximately 85%, while the sequence identity between any two CI genes is approximately 70%. However, the CI genes have evolved in two different groups: the first group (HSP17.6A-I, HSP17.6B-I, HSP17.8-I) has an 84% sequence identity and the second group (HSP17.4-I, HSP17.6C-I, HSP18.1-I) has an 80% sequence identity (**Figure 2**). The sequence identity between any two CI or CII genes is only 30% (**Figure 1**). Genes of both these sHSP classes do not contain introns and are encoded by a single exon. It was previously hypothesized that these two classes of proteins could serve overlapping and redundant functions, however, recent research has shown that this might not be the case.

Few studies have examined the role of CI and CII sHSPs through analysis of null mutants. However, studies with overexpression and genetic knockdown experiments have been performed to investigate possible phenotypic effects resulting from lack of these proteins (McLoughlin et al., 2016). At room temperature (22 °C), both CI and CII were present in transgenic lines engineered to over-express these proteins on a constitutive promoter, while neither the WT nor the RNAi knockdown lines showed detectable protein accumulation. Although these sHSPs were overexpressed, it is important to note that they are expected to be predominantly in their oligomeric form in the absence of stress. However, as heat stress conditions are applied (38 °C), the CI and CII overexpression lines continue to show greater expression of CI and CII sHSPs, respectively, in comparison to WT. Conversely, CI and CII RNAi knockdown lines continued to show dramatically reduced expression of CI and CII sHSPs. Corresponding to their relative expression of CI and CII sHSPs, the overexpression lines exhibited better thermotolerance when compared to WT, while the RNAi knockdown lines exhibited reduced thermotolerance. To test the thermotolerance of these RNAi lines, plants were grown on soil for 10 days and then pretreated at 38 °C for 90 minutes, allowed to recover at room temperature for 2 hours, and then heated at 45 °C for 10 hours (McLoughlin et al., 2016). The sHSP RNAi plants showed severe leaf damage compared to any of the controls, supporting their role in heat tolerance, although these were very severe heat stress conditions. Some studies have also looked at the relationship between CI and CII sHSPs with salt stress. One study found that overexpression of a transgenic CII sHSP from *Primula forrestii* in *A. thaliana* germinated better in the presence of salt stress compared to WT (Zhang et. al., 2018).

CI sHSPs can be observed as dimers, which are thought to be the active form due to the newly exposed hydrophobic residues that can interact with substrates,when the proteins are kept at elevated temperatures. In contrast, CII sHSPs are not observed to dissociate into stable dimers. Rather, they are thought to exhibit chaperone activity through their dynamic subunit exchange, in

which hydrophobic residues would be exposed for substrate binding. Using the binding of a probe for hydrophobic surfaces, bis-Anilinonaphthalene sulfonate (ANS), with CI and CII sHSPs, it was determined that CII sHSPs also exhibit structural changes despite not forming stable dimers. However, CII sHSPs still exhibit temperature-dependent binding to bis-ANS, which means that as temperature increases, more hydrophobic residues are exposed. These hydrophobic residues are expected to be involved in binding unfolded substrate proteins. CI sHSPs exhibit a similar temperature dependent binding to bis-ANS as they also dissociate to stable dimers as temperature increases. In general, the exact mechanism of how sHSPs perform their chaperone activity is unknown and assumed to be mechanistically diverse, involving the dissociation of oligomers into stable dimers, increase in oligomer subunit exchange, and conformational change in structure, all of which are proposed to lead to increased chaperone activity (Basha et al., 2010).

Although CI and CII sHSPs are both localized to the cytosol, they are thought to be structurally and functionally distinct. They are both expressed at different points in development and show maximal expression at different temperatures. Maximal CI sHSP expression was observed at 38-40 °C, while maximal CII sHSP was seen at 42 °C in *Pisum sativum* (Helm et al., 1997). Expression of both classes occurs in pollen tubes, however, only CI sHSPs seem to be expressed more in unpollinated pistils (http://arabidopsis-heat-tree.org/results/iwwqqbrsqz/). Additionally, both in vivo and in vitro analysis of CI and CII sHSPs have shown that these two classes do not form exchange subunits with each other to form hetero-oligomers. There is some indication that the cytosolic sHSPs do not form heteroligomers between classes due to structural differences rather than differences in localization within the cytosol (Hochberg et al, 2018). Although there is a conserved alpha-crystallin domain, there are significant differences in the N-terminal arm and C-terminal tail, which may suggest that these two classes of cytosolic sHSPs have unique functions in plant thermotolerance. The difference in expression patterns and lack of interaction between the two classes suggest that each class may have unique functions.

In addition to being expressed upon heat stress, sHSPs are also expressed in some developmental stages. Consistent with the current paradigm, sHSPs could serve a protective role from proteotoxic stress in periods of development in which expression is rapidly turned on and off for various genes. RNAseq data has shown sHSPs and related transcripts, including HSP70 and heat shock factors are expressed highly in early germination and then rapidly decline (Appendix). In *A. thaliana* specifically, CI sHSPs are expressed abundantly at the mature embryo and dry seed stages. In late stage embryos, developmental arrest of many different physiological processes is needed while maintaining viability and a ready state for germination, which is where sHSPs may play a role. Desiccation tolerance may be provided by sHSPs to allow seeds to dry but still maintain viability. HSP17.4-I expression, in particular, coincides with seed development stages that correspond to dormancy and desiccation tolerance acquisition (Wehmeyer et. al., 1996). Many studies like these have been replicated in other plant species. Additionally, data visualized using http://arabidopsis-heat-tree.org/ indicates high expression of CI and CII sHSPs at the developmental stages of pollen tubes and sperm (**Figure 3**). Overall, it is evident that cytosolic sHSPs are expressed highly in development, however, their specific role remains unclear.

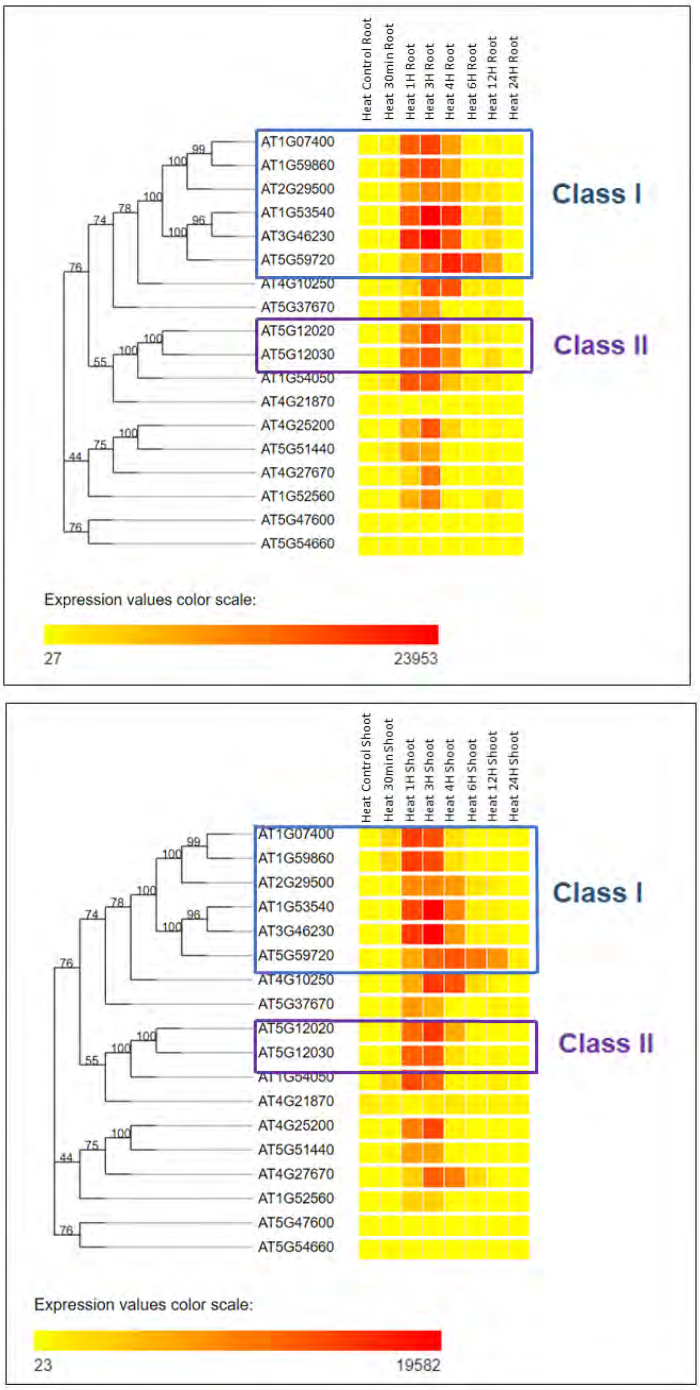

Figure 3. Heat map showing heat-induced expression of CI and CII sHSPs in *Arabidopsis thaliana*

During heat stress, the CI and CII sHSPs are abundantly expressed. All of the genes listed are *A. thaliana* sHSPs. The six CI genes (AT1G07400, AT1G59860, AT2G29500, AT1G53540, AT3G45230, AT5G58720) are labeled in blue. The two CII genes (AT5G12020, AT5G12030) are labeled in purple. Yellow boxes indicate a lower relative expression, while red boxes indicate a higher relative expression. Between 1 and 4 hours, expression of CI and CII sHSPs increases by up to 1000-fold in comparison to the controls for both the root and shoot tissue. AT4G25200

(HSP23.6) encodes a mitochondrial-chloroplast dual localized sHSP and AT1G52560 (HSP26.5) encodes a mitochondrial sHSP. Adapted from http://arabidopsis-heat-tree.org/.

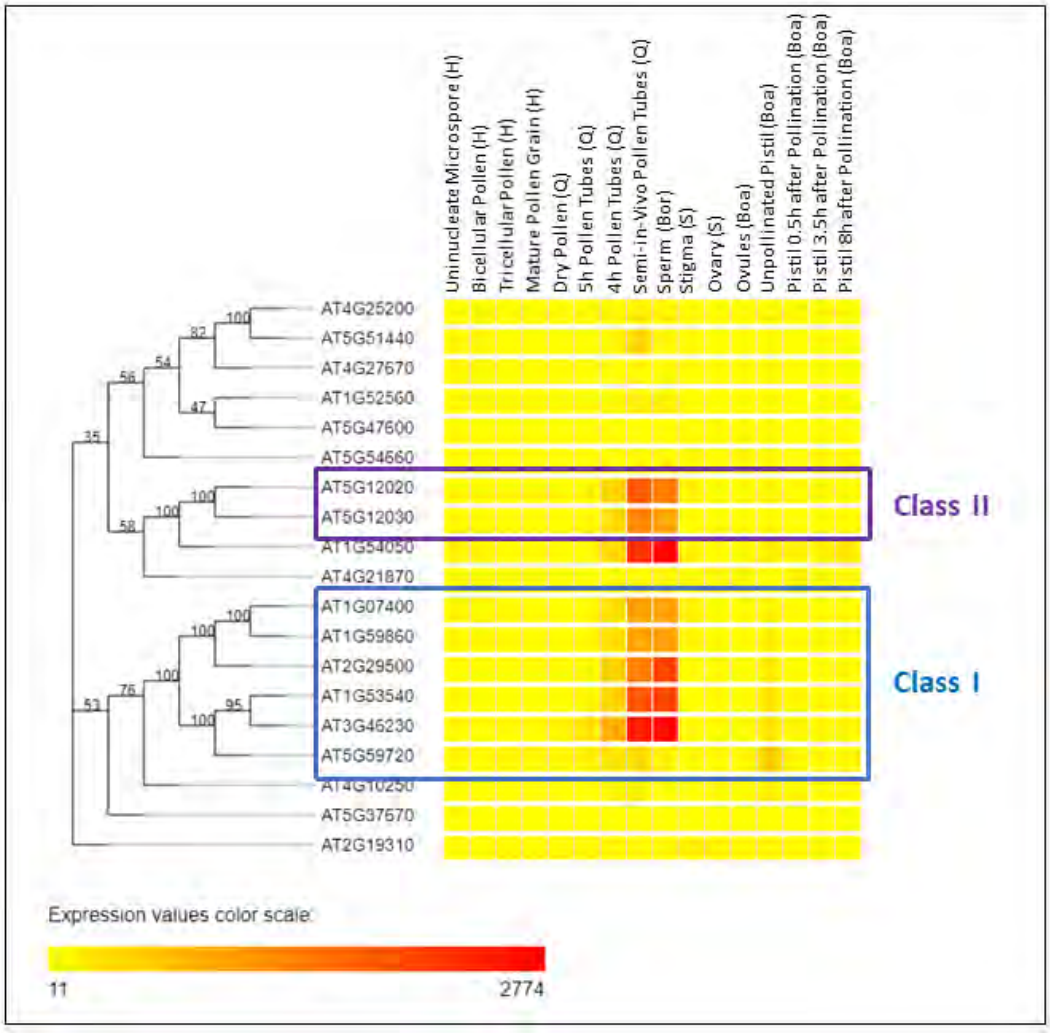

Figure 3. Heat map of upregulated CI and CII sHSPs in *Arabidopsis thaliana* development

The CI and CII sHSPs are highly induced in some developmental stages. All of the genes listed are *A. thaliana* sHSPs. The six CI genes (AT1G07400, AT1G59860, AT2G29500, AT1G53540, AT3G45230, AT5G58720) are labeled in purple. The two CII genes (AT5G12020, AT5G12030) are labeled in blue. Yellow boxes indicate a lower relative expression, while red boxes indicate a higher relative expression. Both CII sHSPs are expressed approximately 1000-fold higher in pollen tubes and sperm. Almost all CI sHSPs are also upregulated at a similar level at these stages. AT4G25200 (HSP23.6) encodes a mitochondrial-chloroplast dual localized sHSP and AT1G52560 (HSP26.5) encodes a mitochondrial sHSP. Adapted from http://arabidopsis-heat-tree.org/.

Although these studies have provided evidence for the role of CI and CII sHSPs in heat tolerance, gene knockouts of the cytosolic sHSPs can help determine whether these proteins are essential to normal growth as well as to thermotolerance. Recently, researchers discovered a gene-editing tool from the bacterium, *Streptococcus pyogenes*, called CRISPR (Clustered Regularly Interspaced Palindromic Repeats). CRISPR uses an enzyme called Cas9 that cuts DNA to produce a double-stranded break, which the cell subsequently repairs. Cas9 is a complex enzyme that has helicase activity, a motif to recognize a PAM (protospacer adjacent motif) sequence, and two nuclease domains, HNH and RuvC (Nishimasu et al., 2014). Cas9 also uses guide RNAs (gRNAs) containing the spacer that acts to specifically locate where the double-stranded break should be made. This protein-RNA complex is then able to target specific genes to knockout or otherwise edit. This will be a useful tool in creating mutants to analyze the effects of sHSP gene knockouts on stress tolerance in *A. thaliana*.

Thesis Overview

The goal of my project is to create, using CRISPR, knockout mutant lines of all CI and CII sHSPs in the model plant *A. thaliana*. The mutant plants will then be examined for defects in normal growth and stress tolerance.

Generation of CII sHSP knockout plants:

I characterized a double mutant of the CII sHSPs in *A. thaliana* to observe any phenotypic effect of a CII sHSP knockout. A mutant carrying a T-DNA insertion in HSP17.6-CII was available for purchase from the Arabidopsis stock center. However, HSP17.7-CII mutants are not available, and even if they were, the two CII sHSPs are linked in a tandem array, which would make it impossible to recover a double mutant by standard genetic crossing. A CRISPR construct was designed to transform the HSP17.6-CII T-DNA mutant to produce a HSP17.6/HSP17.7-CII double knockout. The double knockout was confirmed through PCR genotyping. Immunoblot analysis further confirmed that mutant plants subjected to heat stress did not express CII sHSPs, but the CI sHSPs might be overexpressed to compensate for the absence of the CII sHSPs.

The Cas9 transgene was backcrossed out of the CII sHSP double knockouts plants (13244-8 and 13251-12) to prevent further untargeted mutations that could impact phenotypic analysis of the mutants. Complementation vectors for the two sHSP-CII genes are also cloned to introduce the wild-type genes back into the mutant plants. Analysis of the complemented mutants will confirm that any mutant phenotypes are due to the sHSP knockout mutations. It is expected that the mutants will have a stress-induced phenotypic defect and that the complementation vectors would be able to restore the wild-type phenotype to these mutant plants.

These plants were tested with various assays for differences in heat tolerance compared to wild type plants.

Generation of CI sHSP knockout plants:

CI sHSP knockouts have not previously been made. CI sHSPs are likely structurally similar enough to compensate for each other, making it difficult to find a phenotype of a single mutant. CI sHSP mutant analysis is further complicated because most plants have more than two CI sHSP genes, including *A. thaliana*, which has six CI sHSPs as discussed above. Hence, I designed a CRISPR construct with gRNAs that targets all six CI sHSP genes. After all the gRNAs were cloned into the backbone plant transformation vector, they were transformed into *Agrobacterium tumefaciens* for use in plant transformation. They were used for floral dipping to transform WT plants, and transformants have been isolated that are ready to be tested to identify CI mutants. The same process to backcross the Cas9 gene would have to be employed in these plants before assessing phenotype. It is expected that this cloning-transformation strategy would be able to effectively knockout all CI sHSPs without any significant off-target mutations.

2: Materials and Methods

Plant material and plant growth conditions

All of the plants were kept in a growth chamber at 22 °C with a 16h light/8h dark cycle (40.5 µmol photons m^{-2} s^{-1}). The WT plants that were used for all of the experiments were of the Columbia ecotype. The HSP17.6 T-DNA mutant (SALK_086201.25.80) was obtained from the Arabidopsis Biological Resource Center.

CRISPR-CAS transformation vector:

The pHEC401 vector was used for the transformation into *Agrobacterium tumefaciens* and subsequent floral dipping transformation into *A. thaliana* (Xing et. al., 2014). This vector was specifically chosen as the destination vector for the necessary promoters and genes, most importantly because it contains the EC1.2p egg-cell specific promoter upstream of the zCas9 (zebrafish-codon-optimized Cas9) gene. The EC1.2 promoter regulates transcription specifically during early development of the egg cell, which minimizes the probability of Cas9 to produce "off-target" mutations at undesired locations by ensuring that the Cas9 enzyme is only expressed in a short and specific period. However, every new generation affords the possibility of making new mutations. The egg-cell specific promoter also ensures that the mutant plant does not display a mosaic phenotype, in which some cells are mutated, and others are not; it ensures that the entire plant contains the mutation. The gRNAs are under the regulation of the U6-26P promotor, which drives strong, constitutive expression of the sgRNAs in dicots (Li et. al., 2018). The scaffold at the 5' end of the gRNA is used to anchor the sgRNA to the Cas9 enzyme. This ensures that Cas9 cuts at the precise location with the sequence complementary to the gRNA sequence. The pHEC401 vector also contains a gene that confers resistance to hygromycin, an antibiotic that targets the tRNA acceptor site of the ribosome of both eukaryotic and prokaryotic cells. This antibiotic resistance is used for identifying transgenic plants.

Class II sHSP Guide RNAs:

The CII CRISPR vector was designed by Dr. Indu Santhangopalan and cloned by Alyssa McQuillan in the Vierling lab. The vector contains two gRNAs that both target the HSP17.7-II gene. These gRNAs were designed using the program: http://www.genome.arizona.edu/crispr/CRISPRsearch.html. Once the vector was cloned, it was transformed into *Agrobacterium tumefaciens* and then introduced into *A. thaliana* via floral dipping in the background of the HSP17.6-II T-DNA knockout mutant. This was expected to yield transformed HSP17.6-II mutant seedlings in which the CRISPR/Cas construct would direct mutations in the HSP17.7-II gene.

Plant media and seed sterilization

To screen the CRISPR mutants, the seeds were planted on media with hygromycin. First, the seeds were sterilized in 1.5 mL microcentrifuge tubes using a 50% Milli-Q water, 49.5% Clorox bleach, and 0.5% tween-20 solution. The tubes were vortexed and then incubated for 8 min at 20 °C and 1000 RPM. The tubes were brought to a flow hood where the sterilization solution was

removed. The seeds were washed with Milli-Q water 7 times before the seeds were pipetted onto plates.

MES buffered MS agar media (0.5X Murashige and Skoog medium, 0.5 % sucrose, 0.8% agar, 1.0% 2-(N-morpholino)ethanesulfonic acid) was used for the CRISPR screen. Hygromycin was added to half of the media to a final concentration is 25 μg/mL while the other half of the media served as a positive control and no antibiotic was added. The desired transformants with potential CRISPR mutations possess a hygromycin resistance gene and will be unaffected by the addition of the antibiotic, while the seeds not containing the CRISPR transgene construct will die. The MS media was poured on round petri dishes and the seeds distributed on the plate surface. All of the petri dishes were placed in an enclosed box to stratify for 48 hours at 4 °C. After stratification, the plates were placed in the growth chamber to germinate. When the plants that survived the selection began to grow true leaves, they were transferred onto pots with soil and using forceps. These plants were kept in the growth chamber until they were large enough for genotyping.

Plant DNA extraction

For plant genotyping, Edward's DNA extraction buffer (250 mM NaCl, 25 mM EDTA, 0.5% SDS, 200 mM Tris-HCl pH 7.5) was prepared. One leaf (less than a cm in length) from each 10-day old plant of interest was excised and placed into a 1.5 mL microcentrifuge tube. 150 μL of DNA extraction buffer was added to each sample and the leaf sample was ground using a pestle to disrupt the tissue, producing a green solution. The solution was incubated at room temperature for 5 min before adding 150 μL of isopropanol. The tubes were mixed immediately by inverting and incubated at room temperature for another 5 min. Samples were then centrifuged in a microcentrifuge for 5 min at max speed. The DNA formed a pellet and the supernatant was discarded. 500 μL of 70% ethanol was then added and the tube was centrifuged for another 5 min at max speed. Again, the supernatant was discarded, and the DNA pellet was allowed to dry for an hr to evaporate residual ethanol. The DNA was solubilized using a 50 μL 0.1 X TE buffer (1 mM Tris-HCl pH 8.0, 1 mM EDTA) and kept overnight at 4 °C. These DNA samples were used as template DNA for the polymerase chain reaction (PCR).

PCR Genotyping

PCR amplification of the DNA samples was performed to determine plant genotypes. A master mix for the reactions was made in a 1.5 mL microcentrifuge tube and kept on ice while setting up the reactions for the thermocycler. The master mix contained specific volumes of Mili-Q water, HF Phusion Buffer, dNTPs, Forward Primer, Reverse Primer, and Phusion DNA Polymerase (Table 1). After all of the reagents were added, the master mix was vortexed and centrifuged briefly. Next, 19.6 μL of the master mix was pipetted into 100 μL reaction tubes. Template DNA (0.4 μL) was added to each reaction tube containing the master mix for a final volume of 20 μL (Table 1). The reaction tubes were vortexed and centrifuged briefly again and then placed into the thermocycler.

Table 1. *Polymerase Chain Reaction Volumes and Cycling Conditions*

Components	Volume (μL)	Cycling Conditions
Distilled water	13	3 min – 98 °C 20s – 98 °C | 20s – 63 °C | x39 30s – 72 °C | 3 min – 72 °C Infinite hold – 8 °C
5X HF buffer	4	
10 μM F Primer	1	
10 μM R Primer	1	
10 mM dNTPs	0.4	
Phusion Polymerase	0.2	
Template DNA	0.4	
Final Volume	20	

The results of the PCR were visualized by agarose gel electrophoresis. Agarose, 1 gm in 100 mL of LAB buffer (10 mM lithium acetate, 10 mM boric acid pH 7.5) was added to an Erlenmeyer flask. The flask was heated until almost boiling and swirled to ensure the agarose was dissolved. The solution was allowed to cool for 2-3 min before adding 5 μL gel red. The solution was swirled until it was homogenous, poured into the gel caster and allowed to cool for 60 min.

1X DNA Loading dye (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 each PCR sample. The 1 kb DNA ladder was made using DNA loading dye (1X final concentration), GeneRuler DNA ladder stock (0.1 μg/μL final concentration). The PCR-dye solution (10 μL) and the DNA ladder (2.5 μL) were pipetted into the wells. The gel was run at a constant voltage of 250V for 25 minutes before it was imaged under ultraviolet fluorescence in a GBOX (Syngene).

Leaf heat stress and protein extraction

To obtain protein samples to determine sHSP levels, two leaves were cut from a plant using a forceps and a scalpel, when leaves reached approximately 2 cm in length. The leaves were placed in a round petri dish lined with filter paper, which was wet with 1 mL of milli-Q water. Each plate included a single genotype, with two plates per genotype, one for control and one for heat-stress. The heat stress condition was required because sHSP expression is undetectable at room temperature, but highly induced at 38 °C. For heat stress the plates were put into a dark incubator at 38 °C for 90 min. The control plates were kept at room temperature (RT) in the dark

for 90 min. After 90 min, the heat stress plates were kept with the control plates for an additional 120 min, keeping light exposure at a minimum while transporting the plates. Leaves of each sample were put into a 1.5 mL microcentrifuge tube and frozen in liquid nitrogen. The tubes were stored at -80 °C until protein extraction.

For protein extraction, plant material was kept on ice to prevent protein denaturation. Approximately 150 µL of the protein extraction buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA) was added to the tubes containing the leaves. Plant material was ground using a pestle until the mixture became green and no solid plant tissue remained. The tubes were vortexed for 5 min to ensure the solution was homogenous before centrifuging at 4 °C for 10 minutes at maximum speed. Immediately, the supernatant was removed and transferred to another tube taking care to not disturb the pelleted leaf material. Samples were kept on ice for assaying protein quantity or frozen for later analysis.

Protein Quantification

Quantification of the extracted protein was done using a Bradford assay. A 100 mg/mL stock solution of BSA (bovine serum albumin) was prepared, which was briefly vortexed and kept on a shaker for 5 min to dissolve the BSA. Dilutions were made using the protein extraction buffer to 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL to be used to generate a standard curve. The Bradford dye was diluted with water such that the final concentration is 1X. All samples were vortexed after dilution. For the assay, 200 µL of dye solution was added to each well of 96-well plate then 2 µL of protein solution was added to each well and mixed. The plate was incubated at room temperature for 5 minutes before being inserted into the plate reader (Synergy™ 2 Multi-Mode Microplate Reader, BioTek). The Bradford assay results were used to calculate preparing a final protein sample concentration of 1 µg/µL by addition of 1X dye.

SDS-PAGE

Protein samples were separated using a 15% SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). Since the concentration of the protein samples were 1 µg/µL, 25 µL were added (25 µg in total). 2.5 µL of 1kb GeneRuler protein ladder was added.

Immunoblot analysis:

Immunoblot analysis was performed to confirm that the sHSP mutants were protein null mutants. Blotting paper and nitrocellulose membrane were measured and cut in dimensions corresponding to the SDS gel. The gel rested on top of the nitrocellulose membrane, which was sandwiched between two pieces of blotting paper. The gels and blotting papers were soaked in a transfer buffer and allowed to transfer for 2 hr. After blotting the membrane was stained using Ponceau-S (0.1% in 10% acetic acid) for 1 min. The Ponceau-S background staining was removed by washing with DI water. The membrane was photographed to ensure equal loading based on the intensity of Rubisco (most abundant plant protein) at approximately 55 kDa. The blots were then blocked in a 5% (w/v) milk in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 hr while gently shaking before washing again with TBS-T. The blot was incubated overnight with a 1:1000 dilution of the primary antibody at 4 °C. The blot was rinsed with TBS-

T for 4x5 min while gently shaking. After the washes, 10 mL of TBS-T was added to the petri dish. A 1:5000 dilution of the secondary antibody (ECL anti-rabbit IgG Horseradish-Peroxidase linked whole antibody from Donkey GE Healthcare NA934V) was added to the solution (2 μL) and was incubated for 1 hr while gently shaking. The blot was rinsed again with TBS-T for 4x5 min while gently shaking. As a final step, the blot was incubated with solution A (5 mL 1M Tris pH 8.5, 45 mL H_2O, 110 μL 90 mM p-Coumaric Acid, 250 uL 250 mM Luminol) and solution B (100 uL 30% H_2O_2, 900 μL H_2O) with a 1 mL solution A: 3 uL solution B usage ratio.

Backcrossing to segregate out the Cas9 transgene

Genetic crosses were done to segregate the Cas9 gene and guide RNAs out of the genome of the CII sHSP double knockouts to prevent any further sHSP or off-target mutations. Pollen from a WT male plant was transferred to the stigma of a CII sHSP double-knockout female plant. First, the sepals, petals, and anthers were removed from the female plant using a magnifying glass, forceps, and a scalpel such that the pistil was not damaged. Next, a flower from the male plant was obtained using forceps. The pollen from this male flower was then gently brushed against the isolated pistil of the female plant. Once the stigma has been adequately brushed with pollen, the plant is returned to the growth chamber to allow fertilization. After the pistil developed into a full-sized silique, it was excised. The seeds from the silique were planted on soil to confirm that the backcross was successful by PCR genotyping. These heterozygous plants were then allowed to self-cross. The progeny was genotyped via PCR to identify homozygous mutants not containing the Cas9 gene. All of the lines were successfully backcrossed and then allowed to self cross.

Hypocotyl Elongation Assay of Heat Stress Tolerance

MES buffered MS agar media, prepared as described above, was poured onto square petri dishes and allowed to cool before plating sterilized seeds. Using a pipette, the seeds were plated in horizontal lines such that hypocotyl growth could be measured, and root growth of the row above did not affect the bottom row. The plates were wrapped with micropore tape and stratified vertically in the dark (by wrapping the plates in foil) at 4 °C for 2 days. The plates were moved to a growth chamber for another 2 days at 22 °C to facilitate hypocotyl growth, still kept vertically. Once the hypocotyls were a few millimeters in length, the heat treatment was conducted. For heat stress, plates were kept in a dark incubator for a 90-min acclimation period at 38 °C. Control plates were kept in the dark at room temperature. After the 90-min acclimation period, the heat stress plates were kept with the control plates for an additional 120-min recovery period. After the recovery period, the heat stress plates were exposed to a more acute heat stress condition at 45 °C for 180-210 min. All of the plates were then marked at the tip of each hypocotyl and kept vertically in the dark at 22 °C for an additional 3 days before marking the tip of the hypocotyl again. The plates were scanned and the hypocotyl growth after heat stress was then analyzed.

Root Elongation Assay of Heat Stress Tolerance

The same protocol was used as for the hypocotyl elongation assay with slight adjustments. The acute heat stress period at 45 °C was limited to 120 min, as roots were found to be more heat sensitive. After the heat stress treatment, the roots were marked and measured.

Image Processing and Statistical Analysis

To measure hypocotyl and root growth, the plates were scanned 3 days after the heat stress treatment. These images were analyzed to measure hypocotyl growth using the program Image J. The hypocotyl lengths were compared to a standard measurement to generate accurate lengths. ANOVA and Tukey tests were used to determine if there was a significant difference in hypocotyl lengths between all genotypes.

Light-grown Seedlings Assay of Heat Tolerance

MES buffered MS agar media is prepared as described above. Prior to pouring the media on round petri dishes, plants were divided into eight equal sectors using a marker. The different genotypes to be tested, including a positive and negative control, were each plated twice, using opposite sectors of the plate, in order to have duplicate samples and to control for variation across the plate. Using a pipette, approximately 15-20 seeds were plated in each sector. The plates were wrapped using micropore tape and stratified in a closed box at 4 °C for 2 days. The plates were removed and placed in a growth chamber to germinate and grow for 7 days at 22 °C. After 7 days, the plates were exposed to a heat stress treatment as described above for the hypocotyl elongation assay. The plates were put in a 38 °C incubator for a 90-min acclimation period, then removed and kept at 22 °C for a 120-minute recovery period and put in a 45 °C incubator for a 180-min acute heat stress period. Following the heat treatment, the plates were put in a 22 °C growth chamber for an additional 7 days to recover. Pictures were taken when the differences in phenotype were most apparent.

Seed Setting Assay to Measure Fertility

Seeds were stratified for 2 days on soil at 4 °C in the dark. The pots were then moved to a growth chamber for around 6 weeks. Plants were grown until there were five mature siliques and an inflorescence with many additional flowers. A flower at the stage before pollination was chosen and marked using a thread. To track the siliques, a picture of the entire plant was taken prior to the heat stress. The plants were then exposed to a 4 to 6 hr heat stress at 38 °C in a dark incubator. A set of plants was also kept at room temperature in the dark. A 500 mL beaker with 200 mL of water was placed in the incubator with the plants during the duration of the heat stress to keep humidity levels high. Plants were then returned to the growth chamber to recover for 7 days. Silique lengths above and below the marked silique were removed, photographed and measured.

Design and construction of complementation Vectors for CII sHSPs

To confirm that any phenotype associated with a CII sHSP double-knockout mutant is actually due to the knockout mutations, complementation vectors were designed and transformed in *E.*

coli and *Agrobacterium tumefaciens*. The genes with their native promoters and terminators were cloned into a pREDSEED vector.

First, a plasmid containing the genomic DNA regions of the HSP17.6-II and HSP17.7-II genes was constructed. The HSP17.6-II and HSP17.7-II genes were amplified using the standard PCR procedure outlined above with the primers:

Primer 440 – (5’ TTGAATGTGTATGTTAGAGTTCTCGC 3’)
Primer 441 – (5’ CACGGGTAAGAGAACAAGCTTAGC 3’)

This fragment was then cloned into the pJET1.2 vector with an annealing temperature of 64 °C. The plasmid obtained, p1403, was used for the remaining cloning to obtain the final complementation vectors.

Table 2. *Primers for Cloning Complementation Vectors*

Primers	Sequence (5’ □ 3’)
Primer 463	GGCCGCCATGGCCGCGGGATCACGGGTAAGAGAACAAGCTTAG
Primer 464	GACTCGACTCTAGCTAGTGATGAATGTGTATGTTAGAGTTCTCGC
Primer 465	ACATCGTATGGGTAAGCGACTTGAACTTGTATAG
Primer 466	CGCTTACCCATACGATGTTCCAGATTACGCTTGAGTTTGTTTGTGATCGTG
Primer 467	ATCATCCTTGTAATCAGCAACTTGAACTTGAATTG
Primer 468	GCTGATTACAAGGATGATGATGACAAGTGAGTTTGTTTTGTGATTGTG
Primer 469	CTTCTAAGTTCTTCTTCAAGTCTAGAAGGAGCGACTTGAACTTGTATAG
Primer 470	GAAGAAGAACTTAGAAGAAGACTTACTGAATGAGTTTGTTTGTGATCGTG
Primer 471	CCAATGAGAAACAGCAGCCTTTCTATCAGGAACTTGAACTTGAATTGTCTTTGG
Primer 472	GGCTGCTGTTTCTCATTGGCAACAATGAGTTTGTTTTGTGATTGTG
Primer 473	GGCCGCCATGGCCGCGGGATATCATTGGTGCTATTTAGCAC
Primer 474	GACTCGACTCTAGCTAGTGATCTATATGGGCTTTTAAATTAG

Table 3. *Cloning Strategy for Complementation Vectors*

Plasmid	Description	Cloning Strategy
p1412	pREDSEED-HSP17.6-FLAG/HSP17.7-HA	Template for PCRs: p1403 PCR1: 463+465 ~ 1.7kB PCR2: 466+467 ~ 1.9 kB PCR3: 464+468 ~ 650 bp Gibson assembly of these three PCR fragments with p1068 cut by EcoRV
p1413	pREDSEED-HSP17.6-FLAG	Template for PCRs: p1403 PCR1: 464+468 ~ 625 bp PCR2: 467+473 ~ 1.6 kB Gibson assembly of these two PCR fragments with p1068 cut by EcoRV
p1414	pREDSEED-HSP17.7-HA	Template for PCRs: p1403 PCR1: 463+465 ~ 1.7 kB PCR2: 466+474 ~ 1.2 kB Gibson assembly of these two PCR fragments with p1068 cut by EcoRV
p1415	pREDSEED-HSP17.6-BC2t/HSP17.7-ALFA	Template for PCRs: p1403 PCR1: 463+469 ~ 1.7 kB PCR2: 470+471 ~ 1.9 kB PCR3: 464+472 ~ 625 bp Gibson assembly of these two PCR fragments with p1068 cut by EcoRV

Transformation of Complementation Vectors into E. coli

After complementation vectors were sequenced and confirmed, they were transformed into *E. coli* for storage and later transformed into *A. tumefaciens*. Frozen (-80 °C) Top10 *E. coli* competent cells (Thermo Fisher Scientific) were allowed to thaw on ice for 20-30 min. 5 µL of the Gibson assembly reaction was pipetted into the 50 µL aliquot of the competent cells. The tube containing the competent cells was flicked gently 3 times to get the solution to the bottom of the tube without centrifugation. The cells were kept on ice for 25-30 min, then heat shocked for 30 sec in a 42 °C water bath. Immediately after the heat shock, cells were placed on ice for a 5-min recovery before adding 1 mL of liquid LB media. The tubes were then placed in an incubator at 37 °C to shake at 200 RPM for 90 min. While the tubes were incubating, LB agar plates were prepared in round petri dishes. The LB agar media was melted and cooled slightly before adding the antibiotic spectinomycin to a final concentration of 100 µg/mL. The LB agar

media with the antibiotic is then poured and allowed to cool. Once the media cooled and the 90-minute incubation period for the competent cells was completed, 50 µL of cells were pipetted and spread on the petri dish. Once the petri dishes were dry, they were incubated upside down for 16-18 hr at 37 °C until distinct colonies were visible.

Colony PCR to Test Presence of Complementation Vectors in E. coli

A single, distinct colony from the plates was resuspended in 100 µL of Milli-Q water. 1 µL was used from this mixture for the subsequent colony PCR.

Colony PCR conditions:

Table 4. *Colony PCR Volumes and Cycling Conditions*

Components	Volume (µL)	Cycling Conditions
Distilled water	12	8 min – 98 °C 30s – 98 °C | 30s – 63 °C | x39 90s – 72 °C | 3 min – 72 °C Infinite hold – 8 °C
5X HF buffer	4	
10 µM F Primer	0.6	
10 µM R Primer	0.6	
10 mM dNTPs	0.6	
Phusion Polymerase	0.2	
Template DNA	2	
Final Volume	20	

Cloning of the CI CRISPR Vector

The cloning of the CI CRISPR vector was done by Dr. Patrick Treffon. First, two plasmids, p1430 and p1429, were constructed to contain two different gRNAs each through Gateway cloning (**Table 7**). Each plasmid is expected to have the gRNAs to target all six CI genes once. To clone the two plasmid inserts together, p1430 was first digested with the restriction enzymes, NcoI and SpeI. This cuts the plasmid after the terminator of the second gRNA. The p1429 plasmid was then used as a template for PCR with:

Primer 545: 5’ CGATGAGATAAACCAATACCGACTTGCCTTCCGCACAATAC 3’
Primer 546: 5’ CGCAAATGCTTTTATTCAGTATTGGTTTATCTCATCG 3’

to amplify the region containing the promoters, gRNAs, and terminators. The cut p1430 plasmid and the PCR product from the p1429 plasmid were then cloned using Gibson assembly. The final vector contains the appropriate promoter, gRNA, and terminator for all four gRNAs in sequential order.

Transformation of CI CRISPR Vectors into A. thaliana

To transform the vector into *A. thaliana* via floral dipping, it must first be transformed into *A. tumefaciens*. The p1431 plasmid was transformed into GV3101 competent cells. The GV3101 cells were obtained from -80 °C storage and placed on ice for 10 minutes to thaw. Next, 1 μL of plasmid DNA was transferred to the 50 μL competent cell aliquot. The solution was gently mixed using the pipette before adding to the bottom of a cuvette for electroporation. The cuvette was subjected to 1800V for approximately 5-6 millisec. Immediately, 1 mL of fresh liquid LB media was added for the cells to recover. The GV3101 competent cell and LB mixture was transferred to 1.5 mL epitubes and incubated at 28 °C and 200 RPM for 3 hr. While the cells were incubating, LB agar media with gentamicin and kanamycin (final concentrations: 0.1 mg/mL) was poured onto round petri dishes. 20 μL of cells were streaked onto the LB agar plate. The plates were wrapped in parafilm and incubated at 28 °C for 2-3 days upside down.

A distinct colony was selected to use for an overnight culture (5 mL liquid LB media with gentamicin and kanamycin, final concentrations: 0.1 mg/mL). After incubating for 18 hr at 28 °C, the culture was used for PCR. 100 μL of culture was centrifuged for 2 min at maximum speed. The supernatant was discarded, and the pellet resuspended in 100 μL of Milli-Q water. 1 μL of this solution was used as a template for PCR.

A glycerol stock was made with the *A. tumefaciens* cells containing the p1431 vector (the CI CRISPR vector). A 1 mL storage aliquot was made in a 1.5 mL microcentrifuge tube by mixing 800 μL of 80% glycerol with 200 μL of the overnight culture that was confirmed via PCR. The stock was stored in the -80 °C freezer.

A. tumefaciens carrying the plasmid was grown to obtain a larger preculture for floral dipping transformation into *A. thaliana*. First, a 5 mL overnight culture was made to inoculate the larger preculture using the protocol described above (with the only difference being the glycerol culture was used instead of a colony) and incubated at 28 °C for 18 hr. Next, 300 mL of liquid LB media with gentamicin and kanamycin (final concentration: 0.1 mg/mL) was inoculated with all 5 mL of the overnight culture and incubated at 28 °C for another 18 hr. After growth, the culture was centrifuged at 5000 RPM for 20 min to pellet the cells, and cells were resuspended in 300 mL of freshly made 5% sucrose solution. Slightly before dipping, 100 μL of Silwet L-77 was added to the 300 mL of sucrose for a final Silwet L-77 concentration of approximately 0.03%. Plants were dipped in the sucrose/*A. tumefaciens* solution such that all of the inflorescences were submerged, unsubmerged, and resubmerged for 1-2 min. The dipped plants were then laid down on a tray and covered to maintain high humidity and reduce light exposure. The plants were then moved to a growth chamber and allowed to grow until they were ready for seed collection.

3: Characterizing class II sHSP family knockout mutants

Results and Discussion

Screening for CRISPR mutants of the Class II sHSP genes

The T1 seeds from T0 plants that had been transformed with the CII CRISPR vector were obtained from previous lab members and screened on MS media containing hygromycin. It is expected that the floral dipping transformation of *A. thaliana* will result in approximately 1% of seeds having incorporated the transgene. Therefore, it is necessary to grow seeds on selective MS media containing an antibiotic so that the only plants that grow are those transformed with the antibiotic resistance gene. Seeds that are not transformed are expected to die or have less growth due to the antibiotic. When the seeds were pipetted onto plates, some of the seeds grew well and some did not, as expected. The T1 seedlings that grew on the media were transplanted onto soil. These plants were genotyped to identify mutants. All of the mutants were heterozygotes and kept in a growth chamber for seed collection. The T2 seeds from these plants were planted to genotype T2 lines to identify plants that were homozygous for the transgene (**Figure 4**).

Confirmation of transformants carrying the CII sHSP17.7 mutation

Although the plants that grew on the media likely contain the CRISPR transgene, it was still necessary to determine if the CII HSP17.7 gene was mutated. According to the http://crispr.hzau.edu.cn/CRISPR2/ website, the gRNAs used:

Set One – (5’ GATCTCTGCGGCTTGTAATGA 3’)
Set Two – (5’ GGTGCTCGATAACGTCAGCTC 3’)

had an “on-score” of 0.2951 and 0.2639 for “Set One and “Set Two” gRNAs respectively, which are described in Alyssa McQuillan’s honors thesis. These same gRNAs both had no “off-scores” greater than 0.01. The on-score gives an indication of the effectiveness of a specific gRNA based on a predictive model produced through large-scale empirical analysis (Doench et al., 2014). The scoring system defines an on-score of 0.5-1.0 as being the most effective gRNAs, 0.2-0.5 as being an intermediate effectiveness, and less than 0.2 as being the least effective. Conversely, off-scores defined as 0.5-1.0 pose the greatest risk for unintended gRNA binding, 0.2-0.5 pose an intermediate risk, and less than 0.2 pose a negligible risk. The off-score gives an indication of the probability of a specific gRNA binding to an unintended location also based on a predictive model produced through large-scale empirical analysis. The general equations used to calculate on and off scores that were implemented in the CRISPRv2.0 website are:

Equation 1: $$on(s_j) = \frac{1}{1 + e^{-g(s_j)}}$$

Equation 2: $$off(s_i) = 1 * m_i * p$$

Equation 1 represents the model used to calculate the on-score, with g representing preference for GC content and s_j representing nucleotide preference. A greater value for g and s_j results in a greater value for the on-score. Equation 2 represents the model used to calculate the off-score, with m_i representing gRNA mismatch position, number, and identity and p representing PAM mismatches. A greater value for the m and p results in a greater value for off-score.

Since the on-score for the gRNAs were in the intermediate range of 0.2-0.5, it was still expected that they would be able to bind and produce mutations at the specified locations. This expectation was confirmed via polymerase chain reaction (PCR) (see below).

Five independent sHSP-CII double-knockout mutants have been identified

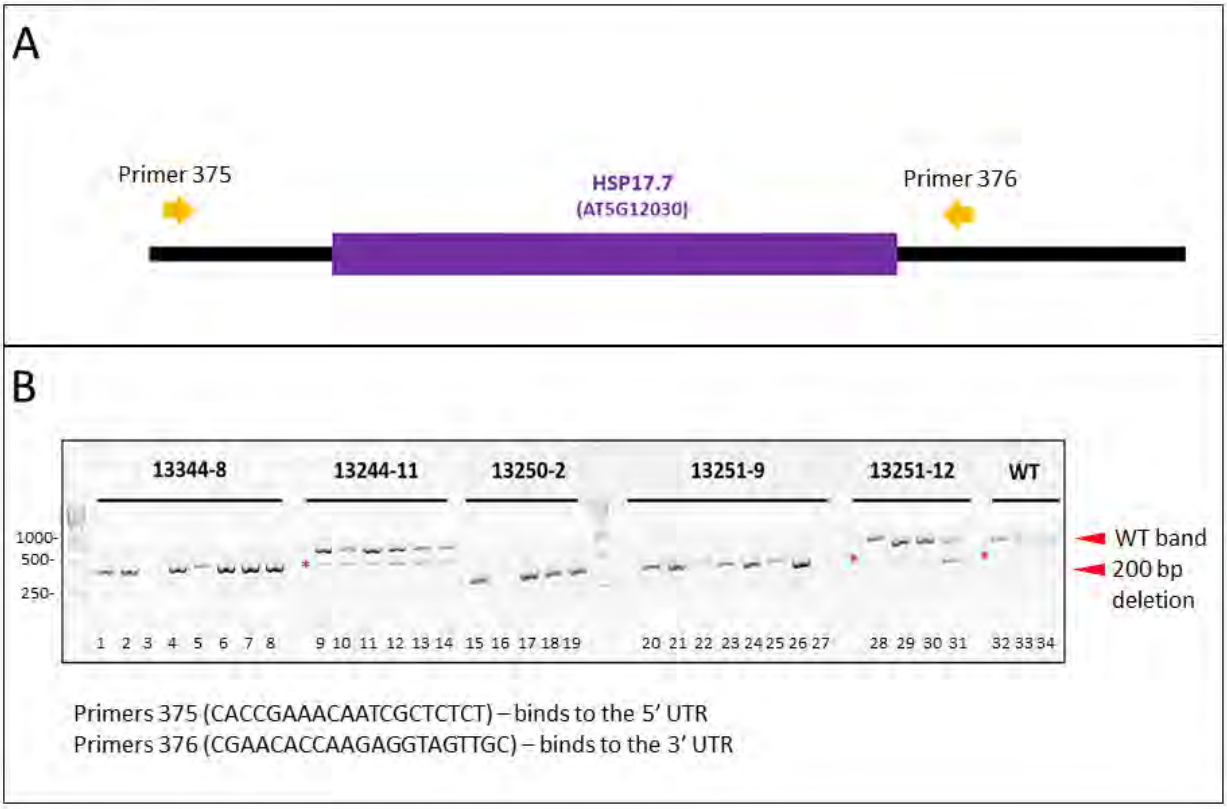

Figure 4. PCR Confirmation of Mutation in HSP17.7 for the CII HSP double-knockouts
(A) The gene diagram is to scale and depicts the location of primer binding sites on the untranslated regions surrounding the coding sequence of HSP17.7
(B) The transformation of the CII CRISPR vector was done in the background of the HSP17.6-II gene. The CII HSP dKO mutants identified are shown to have mutations in the HSP17.7-II gene. Primer 375 binds upstream of the HSP17.7-II coding sequence in the 5' UTR. Primer 376 binds downstream of the HSP17.7-II coding sequence in the 3' UTR. Together, the primers amplify the entire coding sequence and some additional regions surrounding it. Lanes 1-8 show different plants of the same line that have the 200bp deletion. Lanes 9-14 show different plants of the

same line that have a 1bp indel. Lanes 15-17 show different WT plants. The asterisk indicates a non-specific PCR product. Line numbers are defined in Table 5.

It was expected that two gRNAs would target and bind to the HSP17.7-II gene and result in a deletion of some nucleotides. Therefore, the mutants were screened using PCR with primers that bind to the 5' UTR and the 3' UTR of the HSP17.7-II gene. Any deletion or mutation of the coding sequence of the gene is then amplified. Deletions can be detected by gel electrophoresis based on a difference in size of the PCR product compared to that from the WT, however, point mutants could only be identified by sequencing. A band of ~680 bp is indicative of the WT HSP17.7-II gene. A band of similar size could also carry a point mutation or small indel. The band at ~500 bp represents a deletion in the HSP17.7 gene (**Figure 4B**). In total 5 independent lines of CII HSP dKO mutants were identified, 3 of which had a 229 bp deletion in the coding sequence of the HSP17.7 gene. The other 2 mutants identified had point mutations that resulted in a premature stop codon of the HSP17.7 gene (**Figure 5**).

Table 5. *CII sHSP double-knockout lines*

Type of Mutation	Name of Line	Designation on Figures
Deletion	13244-8	200bp deletion
Deletion	13251-9	200bp deletion
Deletion	13250-2	200bp deletion
Indel mutation	13244-11	1bp Indel
Indel mutation	13251-12	1bp Indel

Figure 5. Coding Sequences of the HSP17.7-II Indel Mutants
(**A**) Deletion of an adenine at position 312 of the coding sequence in plant line 13244-11 introduces a stop codon 64 base pairs downstream of the mutation. This results in a gene that could only produce a truncated protein of 74 amino acids, while the WT protein is 157 amino acids. This means that a portion of the alpha-crystallin domain and all of the C-terminal extension of the protein would be deleted.
(**B**) Insertion of an adenine at position 540 in plant line 13251-12 introduces a stop codon one codon downstream of the mutation. This results in a truncated protein with 129 amino acids. This means that a small portion of the alpha-crystallin domain and all of the C-terminal extension would be deleted.

The 13244-11 point mutant has a deletion of an adenine at position 312 (which is 157 bp into the coding region) and also an addition of an adenosine at position 540 (which is 384 bp into the coding region), resulting in a premature stop codon at amino acid 74. The insertion corresponds to the location that the gRNA from Set_Two binds, while the deletion corresponds to the location that the gRNA from Set_One binds (Alyssa McQuillan's Honors Thesis).

The 13251-12 point mutant has an insertion of an adenine at position 540 (which is 384 bp into the coding region), resulting in a premature stop codon at 126 AA. The deletion corresponds to the location that the gRNA from Set_One binds (Alyssa McQuillan's Honors Thesis).

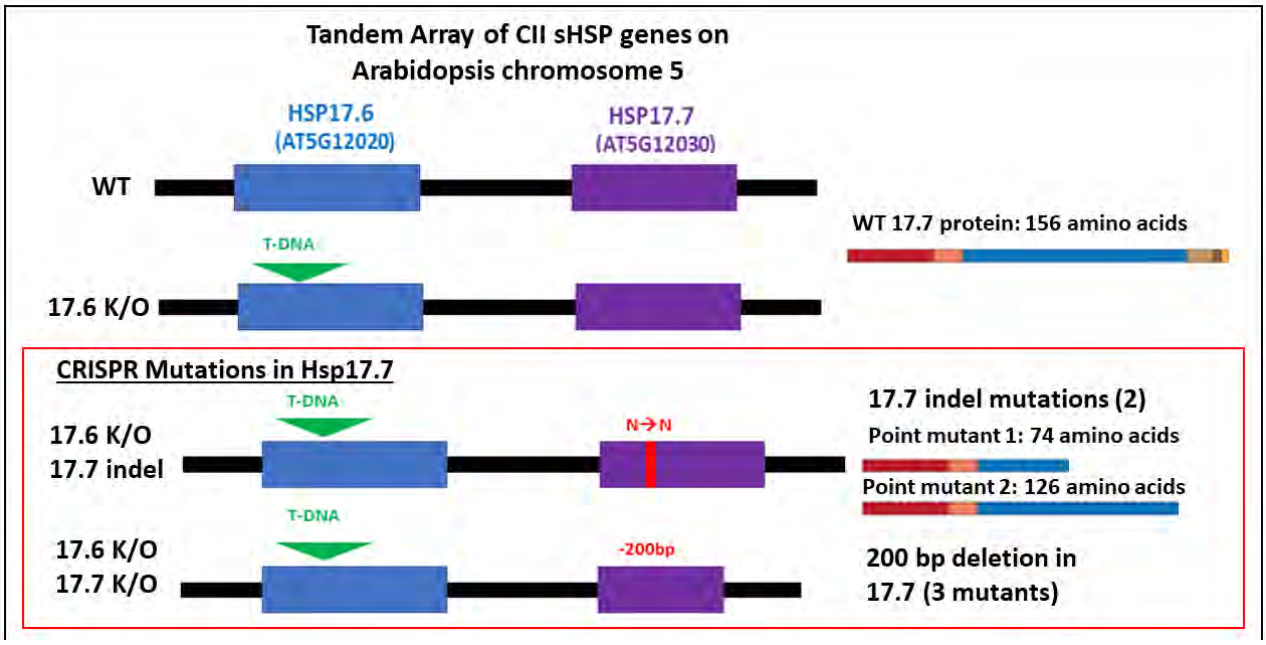

Figure 6. Gene diagrams of CII HSP double-knockout lines identified

Both the HSP17.6 (AT5G12020) and HSP17.7 (AT5G12030) are on chromosome 5 and are approximately 850 base pairs apart. The wild-type HSP17.6-II coding sequence is 468 base pairs long. The wild-type HSP17.7-II gene coding sequence is 471 base pairs long. The 1bp indel in the HSP17.7 gene has the same coding sequence length as WT. The 229bp deletion leads to a shorter HSP17.7 coding sequence of 271 bp.

Immunoblot analysis confirms sHSP-CII mutants are protein nulls

Once the mutants were identified via PCR genotyping, they were tested using immunoblot analysis to determine the level of CI and CII sHSP expression. In total, five different genotypes were used: wild-type, a 17.6-II T-DNA knockout line, and the three CII sHSP double-knockout mutants with a deletion in the HSP17.7-II gene (Table 5).

Two different antibodies were used to detect the presence of any CII sHSPs. The UAZ44 antibody binds to HSP17.6-II, but detects any CII sHSP. The UAZ45 antibody binds to a yeast HSP26 recombinant protein, but detects any CII sHSP. Leaves removed from plants of each of the lines were either maintained at room temperature or heat stressed to allow accumulation of HSPs (Materials and Methods) before protein extraction. In the room temperature controls, immunoblots with either antibody showed no band around the expected size for CII sHSPs (17

kDa), suggesting that there was virtually no expression at room temperature. WT had greater expression of CII sHSPs than the HSP17.6-II single mutant (**Figure 6A**). This matches the expectation that the HSP17.6-II single mutant only expresses HSP17.7-II and does not express HSP17.6-II. There is no expression of either CII sHSP in the double-knockout mutants, confirming that the CII sHSP double-knockouts are protein null mutants (**Figure 6A**). In Figure 6B, the bands are distinct enough to distinguish the upper HSP17.7-II band from the lower HSP17.6-II band. This also makes it possible to see that the HSP17.6-II single knockout has only the upper HSP17.7-II band as expected, because it is the only CII sHSP being expressed in the mutant (**Figure 6B**).

Two different antibodies were used to detect the expression of CI sHSPs using the same samples. The UAZ14 antibody was generated against HSP17.6-I, but detects any CI sHSP. The UAZ15 antibody was generated against the C-terminus of HSP17.6-I, but also detects any CI sHSP (Helm et. al, 1997). Both immunoblots are presented because the first gel had a weaker signal, and did not show any expression in room temperature controls as expected (**Figure 7A**). The second gel had a stronger signal, and unexpectedly showed expression in room temperature controls. Since the CI and CII sHSPs are both similar and localized to the cytosol, it is possible that there is an increase in expression of CI sHSPs in response to the lack of CII sHSPs. This concept was also examined in a previous study looking at RNAi knockdown lines. Although there was no compensation in the CI and CII sHSP RNAi transgenic lines (McLaughlin et al, 2016), there may be compensation in the CII sHSP knockout mutants (**Figure 7B**). The level of CI proteins after heat stress appears higher in the knockout mutants than in the WT or the 17.6-CII knockout.

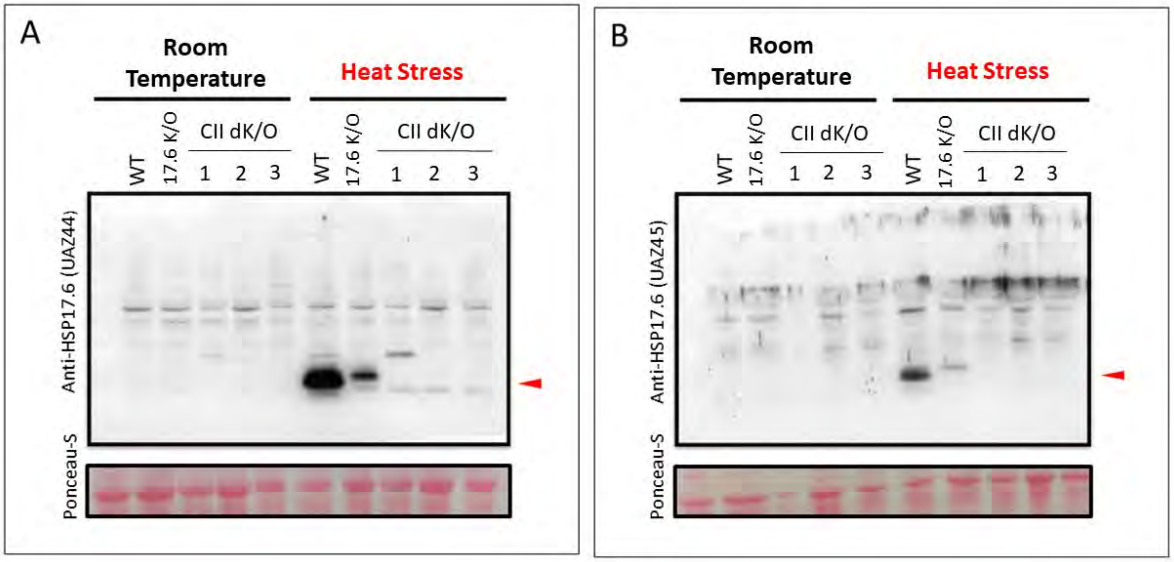

Figure 7. Immunoblot confirmation of CII HSP double-knockouts as null mutants.

Total protein was extracted from leaves maintained at room temperature or subjected to a heat stress treatment. Equal concentrations of protein were separated by SDS-PAGE and transferred to nitrocellulose. The specific antibodies used for each immunoblot are indicated above. Ponceau-S staining shows Rubisco as a loading control. The red arrow indicates the size at which CII HSPs are present. The CII dK/O 1, 2, and 3 refer to the three 200bp deletion mutants. **(A)** The antibody detects both CII sHSPs and shows that CII sHSP double-knockouts are not expressing any CII sHSP. **(B)** The antibody also probes for both CII sHSPs and shows that the upper band is HSP17.7-II which is present in the HSP17.6 single mutant but not the CII double mutant.

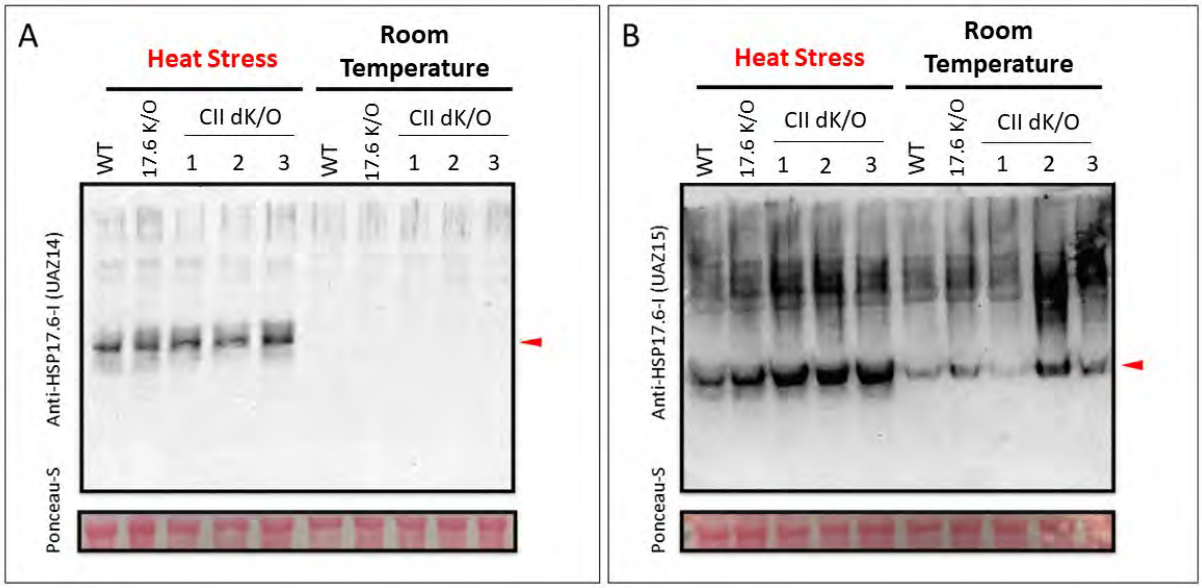

Figure 8. Immunoblot analysis of CI HSPs expression in CII HSP double-knockouts

(A) The antibody probes for all CI sHSPs, which are only expressed after the heat stress treatment and not at room temperature. **(B)** The antibody also probes for all CI sHSPs. The mutants look to have different expression levels for the CI sHSPs in comparison to WT. Sample preparation and separation as in Figure 7.

Backcrossing to segregate out the Cas9 gene in the CII sHSP double-knockouts

The CII sHSP double-knockouts were backcrossed to WT to remove the Cas9 transgene and guide RNAs from the genome. Since the enzyme was expressed using an egg-cell specific promoter, every new generation would result in transient expression of Cas9, which could allow for potential additional mutations, including off target mutations. Two important loci were considered in this cross, the CII sHSP genes and the Cas9 gene. The CII sHSP genes were considered as one locus because they are less than 1 kb apart on chromosome 5, so that they effectively segregate together. Each homozygous CII sHSP double-knockout mutant was backcrossed to a WT plant, resulting in F1 plants that are all heterozygous for both the CII sHSP genes and the Cas9 gene (**Figure 9**). These heterozygous plants were then allowed to self-pollinate to produce F2 plants. It was expected that 1/16 plants would be homozygous for the CII sHSP double-knockout and have no Cas9, which were the plants selected for further experiments (**Figure 9**).

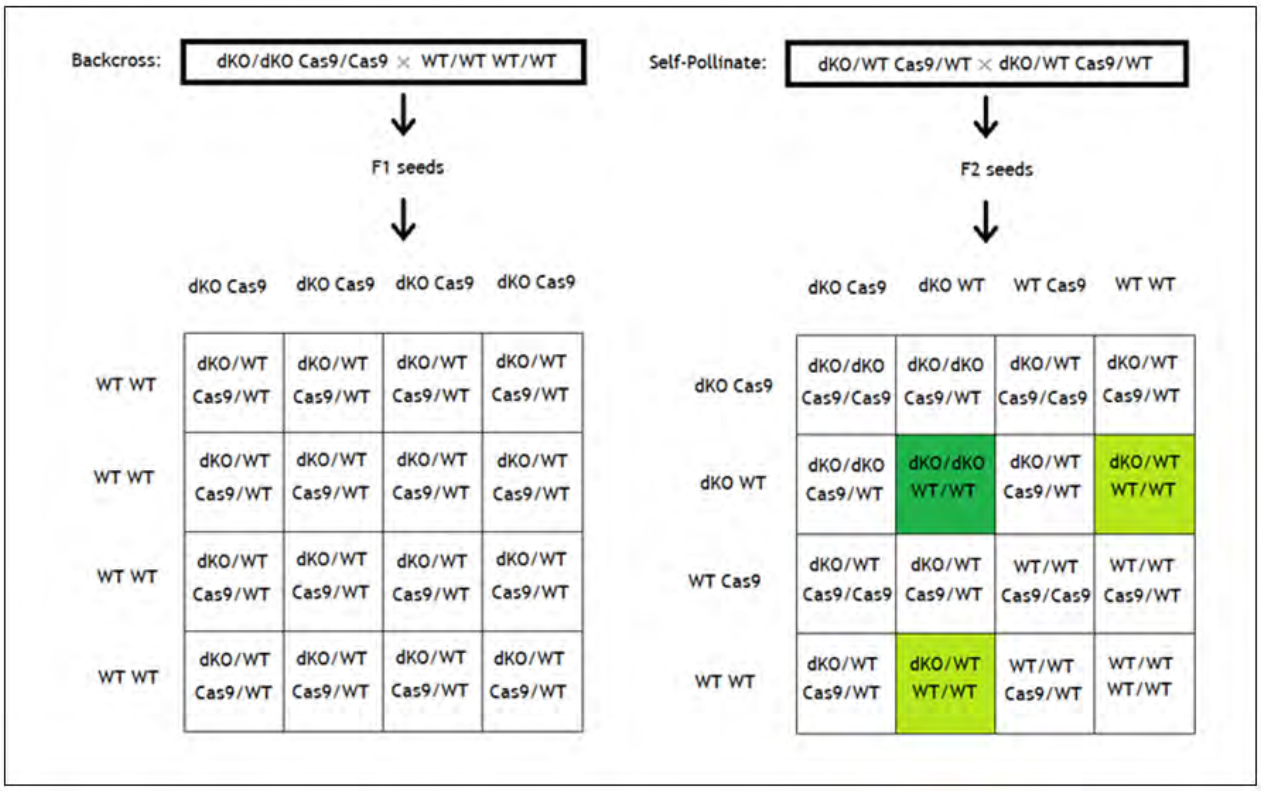

Figure 9. Expected Mendelian segregation from a CII sHSP double knockout backcross.

From the initial backcross, all of the F1 progeny are expected to be heterozygous for both loci (left). These heterozygous plants were allowed to self-pollinate. From the F2 progeny, only 1/16 of the plants were expected to be homozygous for the CII sHSP double-knockout and not contain Cas9 (right – dark green square). Plants lacking Cas9 and heterozygous for the dKO (light green squares) would also be obtained and could be propagated to the next generation to obtain dKO knockouts.

The F2 plants from two different CII sHSP double-knockout lines were genotyped using PCR. Three different primer pairs were used to genotype the plants (**Table 6**). Primers 197+349+350 were used to amplify the HSP17.6 gene, yielding an 850 bp product for WT and a 1 kb product for the T-DNA mutants. In this three-primer combination, the T-DNA primer (197) binds to a region in the T-DNA insertion and amplifies a region, including part of the HSP17.6 gene, with primer 350 for the T-DNA mutant. Since WT has no T-DNA insertion, the T-DNA primer does not bind anywhere and only primers 349+350 bind to amplify part of the HSP17.6 gene. Primers 377+378 were used to amplify the HSP17.7 gene, yielding a 650 bp product for WT and a 450 bp product for the deletion mutants. Since these deletion mutants were produced by the simultaneous binding of two gRNAs 200 bp apart, the gene is 200 bp shorter in the CRISPR mutants. Primers 585+586 were used to amplify a region spanning part of the zCas9 gene and the corresponding EC1.1 promoter, yielding a 936 bp product for the CRISPR mutants and no

product for WT. This also makes sense because the WT plants do not have the CRISPR vector in their genome, while the CRISPR mutants do. The results of the PCR were then visualized on an agarose gel.

Table 6. *Primers for genotyping F2 CII sHSP double-knockout mutants*

Primer	Sequence (5′ → 3′)	Purpose
197	ATTTTGCCGATTTCGGAAC	T-DNA primer for characterizing SALK T-DNA insertion lines
349	TTCACAAAAACCAGAACGACC	LP to genotype HSP17.6-II T-DNA mutant with primer 197
350	ACCAATTGGTCTGTCACTTGG	RP to genotype HSP17.6-II T-DNA mutant with primer 197
377	ACATGCTTGAAGCCCCTGAA	LP (binds in the coding sequence) to genotype HSP17.7-II with primer 378
378	GCATGGATGGTTCAAGAGAGC	RP (binds in the 3' UTR) to genotype HSP17.7-II with primer 377
585	GTACTCGATCGGCCTCGATATTGG	RP to genotype CRISPR mutants, binds in zCAS9 coding region
586	GGAGCCTTCGTAATCTCGGTGTTC	LP to genotype CRISPR mutants, binds in the EC1.1 promoter region

Design of dCAPs Markers for Genotyping the Indels

Since there are single nucleotide changes in the point mutants, these mutants would result in a similar sized band to WT. To determine whether the gene was WT or mutant, dCAPs (derived Cleaved Amplified Polymorphic Sequences) markers were designed using http://helix.wustl.edu/dcaps/dcaps.html. These dCAPs markers, in conjunction with the single nucleotide mutation, produce a restriction site on the mutant but not WT when the DNA is amplified with the dCAPS primers. Since the specificity of the dCAPs marker depends on the specific nucleotide change, two different dCAPs markers were designed for each of the two lines carrying point mutations (13244-11 and 13251-12).

The 13244-11 point mutant has a deletion of an adenine at position 312 (157 bp into the coding region) and also an addition of an adenine at position 540 (384 bp into the coding region), resulting in a premature stop codon at amino acid 74. The initial insertion corresponds to the

location that the gRNA from Set_Two binds while the deletion corresponds to the location that gRNA from Set_One binds (Alyssa McQuillan's Honors Thesis).

The 13251-12 point mutant has an insertion of an adenine at position 540 (384 bp into the coding region), resulting in a premature stop codon at amino acid 126. The deletion corresponds to the location that the gRNA from Set_One binds (Alyssa McQuillan's Honors Thesis).

To determine if there is a point mutation that occurred exclusively in the 13244-11 line (the A-X mutation at position 312), PCR is done using plant genomic DNA as a template. The dCAPs marker primer (5' TCCACGGCGAAAACGTACGCATCCcGG 3') and primer 377 (Table 6) is used to amplify a region flanking the point mutation. The PCR product is then incubated with the restriction enzyme SmaI. The PCR product from the mutant would have a SmaI restriction site resulting in a 113 bp and 24 bp fragment when cut with SmaI. The WT PCR product would not have a SmaI restriction site resulting in a single 137 bp fragment.

To determine if there is a point mutation that occurred in the 13251-12 line (the insertion of an A at position 540), PCR is done using the plant genomic DNA as a template. The dCAPs marker (5' CAGATTTGGAGAAGATCTCTGCGGCTTtTAA 3') and primer 376 is used to amplify a region flanking the point mutation. The PCR product is then incubated with the restriction enzyme DraI. The PCR product from the mutant would have a DraI restriction site resulting in a 156 bp and 29 bp fragment. The WT PCR product would not have a DraI restriction site resulting in a single 183 bp fragment.

These dCAPs markers are designed and ready to use for future experiments.

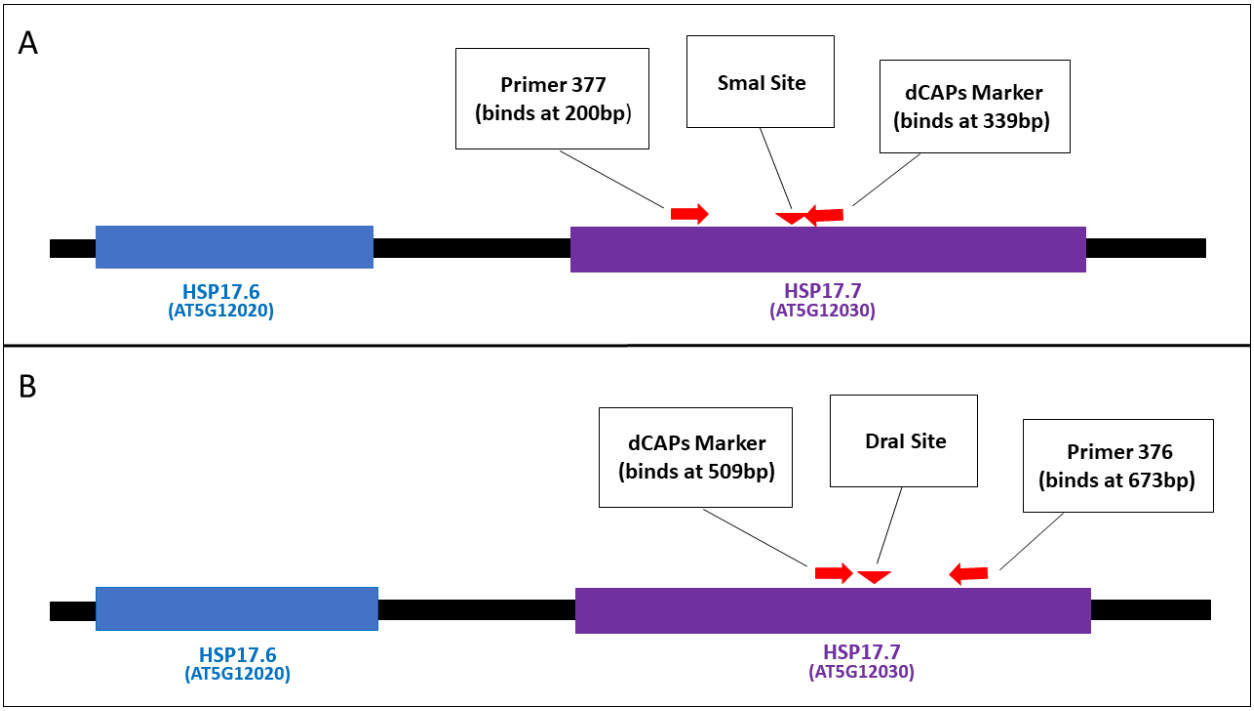

Figure 10. Gene diagram of dCAPs markers to genotype point mutants

Of the five independent CII sHSP double-knockout lines identified, two had point mutations in the HSP17.7-II gene. To screen for each of these mutations, dCAPs markers were designed. (**A**) For the first point mutant (13244-11), PCR amplification with primer 377 and the dCAPs marker and subsequent digestion with SmaI allows for differentiation of the mutant and WT gene. (**B**) For the second point mutant (13251-12), PCR amplification with primer 376 and the dCAPs maker and subsequent digestion with DraI allows for differentiation of the mutant and WT gene.

Hypocotyl Elongation Assay of dKO heat sensitivity

To test heat sensitivity of the mutants, a hypocotyl elongation assay was performed (Kim et. al., 2017). Four different genotypes were tested using: WT, *hot1-3*, 13244-8 (CII dKO containing a 200bp deletion in the HSP17.7 gene) and 13244-11 (CII dKO containing a point mutation in the HSP17.7 gene). The *hot1-3* seedlings are null mutants for the molecular chaperone Hsp101 and are highly heat sensitive; they serve as a heat-sensitive control (Hong et. al, 2003). It was expected that these seedlings would show little to no hypocotyl elongation after heat stress. Using ANOVA and Tukey tests, only *hot1-3* showed a statistically significant difference from WT; it elongated significantly less after heat stress as expected. The two dKO mutants grew like WT (**Figure 11**).

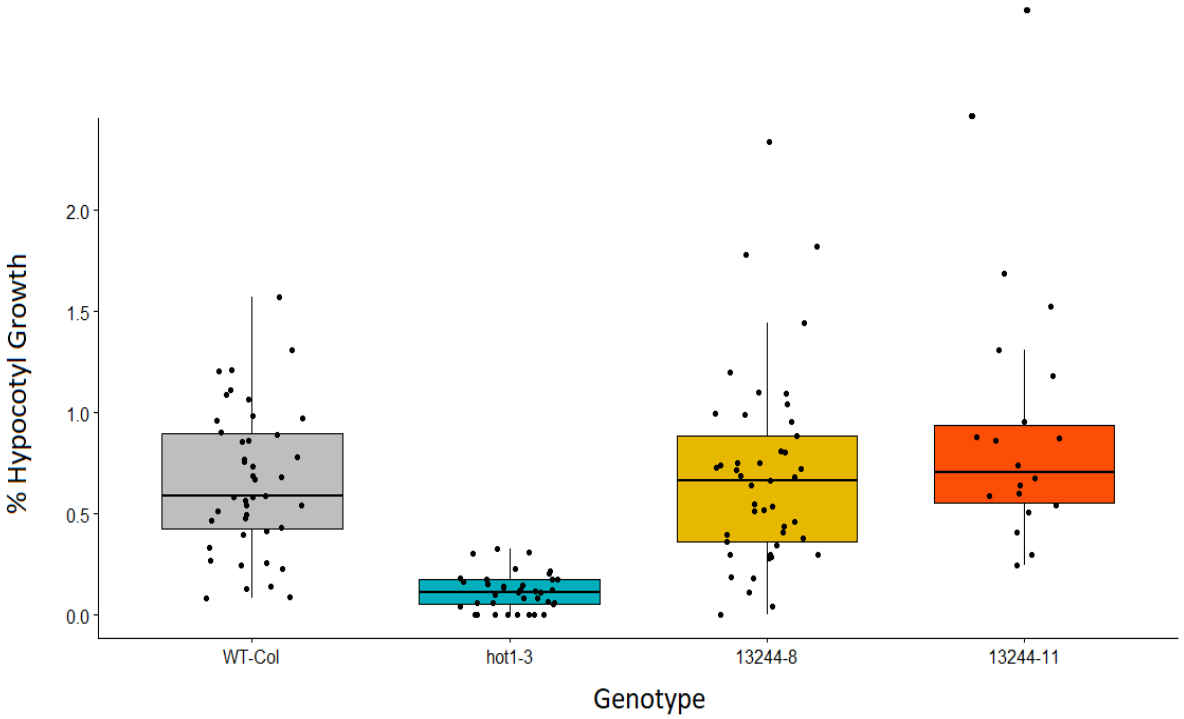

Figure 11. Percent Hypocotyl Growth After Heat Stress

Boxplot hypocotyl growth after heat stress divided by hypocotyl growth in the control condition is shown for each genotype. There were 44 WT seedlings, 35 *hot1-3* seedlings, 46 13244-8 (CII HSP dKO with a 200bp deletion in the HSP17.7-II gene) seedlings, and 18 13244-11 (CII HSP dKO with a point mutation in the HSP17.7-II gene) seedlings. Seedlings that did not germinate were omitted. All of the data collected was from a single experiment. The horizontal line in each boxplot represents the mean. The whiskers represent the interquartile range. The individual measurements are shown as dots. The graph was created using R studio.

Heat tolerance of root growth

In addition to the hypocotyl elongation assay, a root growth assay was performed to test the heat sensitivity of the mutants. The same 4 genotypes were also used in this assay: WT, *hot1-3*, 13244-8, and 13244-11. The *hot1-3* seedlings again served as a heat-sensitive control. It was expected that these seedlings would show little to no root elongation after heat stress. The two CII double-knockout mutants were compared to the WT control and heat-sensitive control, *hot1-3*. Using ANOVA and Tukey tests, there was a significant difference when comparing any two genotypes (**Figure 12**).

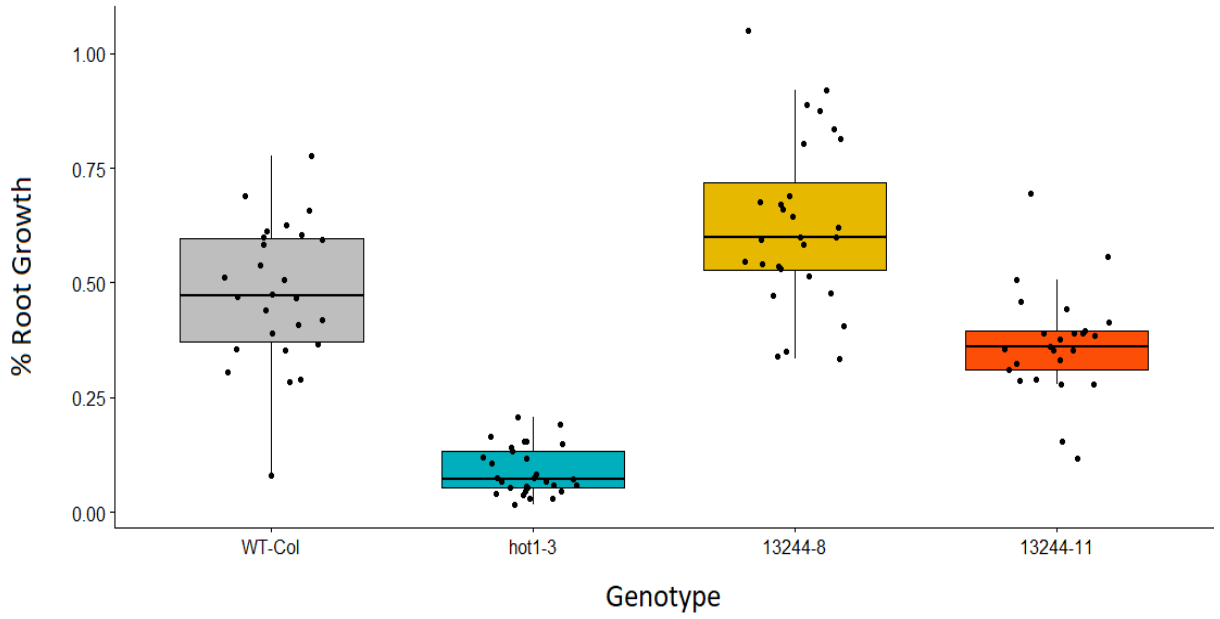

Figure 12. Percent Root Growth After Heat Stress
Boxplot showing results of the heat stress as root growth during heat stress divided by the average root growth in the control condition is shown for each genotype. The horizontal line in each boxplot represents the mean. The individual points are also displayed as dots. The graph was created using R studio. Three replicate plates were heat stressed all on the same day and same time to obtain the data.

Heat Tolerance of Light Grown Seedlings

As an additional way to test the heat sensitivity of the mutants, plants were grown on plates in the light. The four genotypes used were: WT, *hot1-3*, 13244-8, and 13244-11. The *hot1-3* seedlings were expected to be heat-sensitive and have a chlorosis phenotype after heat stress. In comparison to WT and the heat-sensitive control, it may be that the CII double-knockout mutants have an intermediate phenotype. Some of the CII HSP double-knockout mutants showed smaller and less green seedlings after the heat stress in comparison to WT. However, more experiments need to be conducted to confirm this data (Kim et al, 2017).

Developing a Seed Setting Assay to Measure Fertility During Heat Stress

An assay was developed to determine if there is any defect in fertility when plants are heat stressed at key developmental stages. An assay of this type was of interest given evidence that these sHSPs are expressed in germinating pollen (**Fig. 3**). The assay was developed with WT plants to determine the correct parameters that would give an indication of a phenotype. It was expected that plants heat stressed for a longer time would show a greater reduction in fertility than plants stressed for shorter times. Plants stressed for 6 hours would have the shortest siliques

at key developmental stages, plants that were heat stressed for 4 hours would have slightly shorter siliques at these stages, and plants that were at room temperatures would have no reduction in silique length. These expectations were confirmed in the experiment. The marked silique was between stage 12 and 13, and it was termed “Silique 0” (**Figure 13**). Further measurements on seed number and viability could also be examined in future experiments.

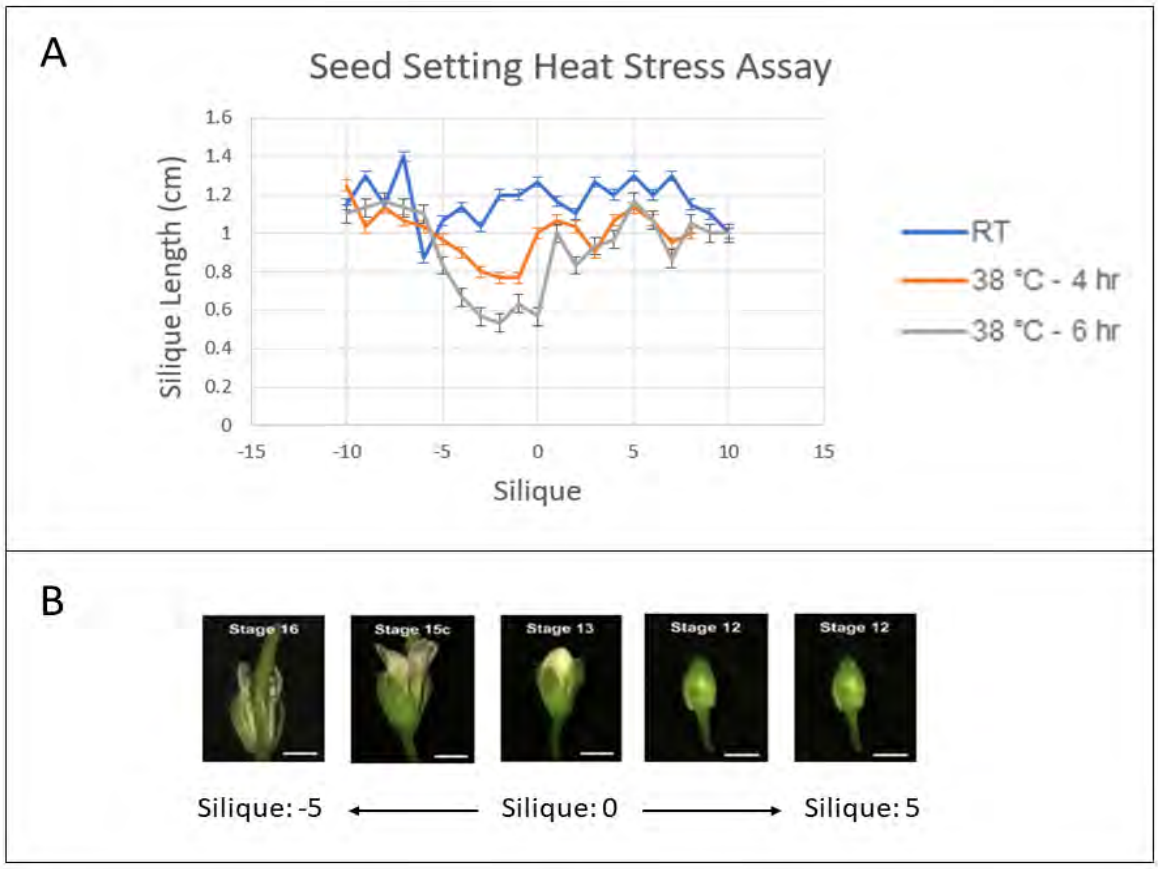

Figure 13. Silique Length of WT Plants After Heat Stress

Three plants were tested for each condition (RT, 38 °C for 4h, 38 °C for 6h) on the same day at the same time. (**A**) Average silique length at flower positions relative to the flower marked prior to heat stress “Silique 0”. Three WT plants each were either maintained at room temperature or heat stressed for 4 or 6 hr after marking Silique 0. Silique length was measured 7 days following the heat stress. The plants were grown in a growth chamber at 22 °C with a 16h light/8h dark cycle. (**B**) Flower developmental stages during the heat stress treatment. Flowers that developed into siliques 0-5 were between stages 13 and 12, while flowers that developed into Siliques -5-0 were between stages 16 and 13.

Cloning of CII Complementation Vectors

Vectors to complement the double knockout mutant lines were designed by Dr. Patrick Treffon, and I completed the vector construction. The CII sHSP genes were cloned with their native promoter and terminator to ensure that the mutants are complemented with the same level of functional protein as in WT. Each of the complementation vectors contained an affinity tag recognized by a specific antibody or nanobody attached to the C-terminal end of the protein. Primers 440 and 441 were used to amplify the entire region encompassing both genes. Primer 440 binds 578 bp upstream of the HSP17.6-II coding sequence, while primer 441 binds 1136 bp downstream of the HSP17.7-II coding sequence. This PCR product was cloned into p1403, a pJET1.2 vector.

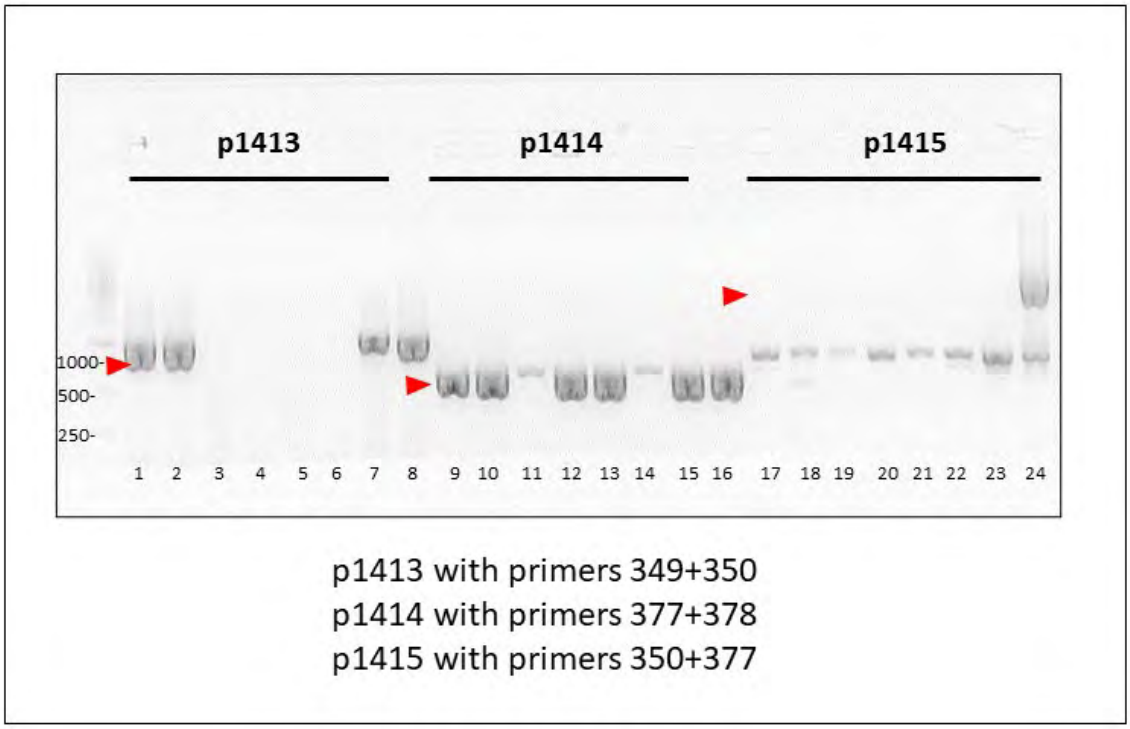

Figure 14. PCR Confirmation of Complementation Vector Cloning

In total, four complementation vectors were cloned: p1412, p1413, p1414 and p1415. p1412 was sequenced and confirmed to contain the gene. After transformation into *A. tumefaciens*, a single colony was used as template DNA for PCR. p1413 contains the HSP17.6-II gene (lanes 1, 2, 7, and 8). p1414 contains the HSP17.7-II gene (lanes 9-17). p1414 contains both CII sHSP genes as seen in lane 24. The red arrows indicate the expected size for positive transformants.

Table 7. *Cloning Strategy for Complementation Vectors*

Plasmid	Name	Description
p1412	pREDSEED-HSP17.6-FLAG/HSP17.7-HA	Native promoter, coding sequence HSP17.6, FLAG tag, native terminator. Native promoter, coding sequence HSP17.7, HA tag, native terminator.
p1413	pREDSEED-HSP17.6-FLAG	Native promoter, coding sequence HSP17.6, FLAG tag, native terminator.
p1414	pREDSEED-HSP17.7-HA	Native promoter, coding sequence HSP17.7, HA tag, native terminator.
p1415	pREDSEED-HSP17.6-BC2t/HSP17.7-ALFA	Native promoter, coding sequence HSP17.6, BC2t tag, native terminator. Native promoter, coding sequence HSP17.7, ALFA tag, native terminator.

Regions from this p1403 vector were then amplified using PCR and cloned into the pREDSEED backbone vector to produce the final complementation vector constructs (**Table 7**). The native promoter for HSP17.6-II was defined as 1337 base pairs upstream of the coding sequence and the native terminator was defined as 577 base pairs downstream of the coding sequence. The native promoter for the HSP17.7-II gene was defined as 965 base pairs upstream of the coding sequence and the native terminator was defined as 1337 base pairs downstream of the coding sequence. This was then used as a template for PCR, with one of the primers containing the sequence for the different affinity tags.

The complementation vector cloning and transformation was confirmed in *E. coli* using PCR with primers that amplified the HSP17.6 and HSP17.7 genes. Primers 349+350 were used to amplify the HSP17.6 gene in the p1413 vector because only the HSP17.6 gene is present. Primers 377+378 were used to amplify the HSP17.7 gene in the p1414 vector because only the HSP17.7 gene is present. Primers 350+377 were used to amplify both the HSP17.6 and HSP17.7 gene in the p1415 vector to confirm the presence of both genes (**Figure 14**).

Conclusions and Future Directions

Five independent CII sHSP double-knockouts mutants, in which both HSP17.6-II and HSP17.7-II genes are knocked out, were identified and confirmed. All five have a T-DNA insertion in the HSP17.6-CII genes and CRISPR created mutations in the adjacent HSP17.7-CII gene. Three of the five CRISPR created 17.7-CII mutations are 200 bp deletions, while two are point mutations. The three CII double-knockouts that have the 200 bp deletion in the Hsp17.7-CII gene (plants 13244-8, 13250-2, 13251-9) were found to be null mutants, showing no expression of CII sHSPs. These are the first plants to be characterized that lack this class of cytosolic sHSPs. The two CRISPR mutants having only point mutations in Hsp17.7-CII have not yet been tested for sHSP expression. These point mutations created premature stop codons in the genes, such that partial proteins may accumulate. This remains to be tested.

Immunoblot analysis indicates that the three CII double-knockouts with the 200 bp deletion in Hsp17.7-CII may have increased sHSP-CI expression. This potential upregulation of CI proteins in the CII mutants may be compensation for the lack of the CII HSPs. This may mean that CI and CII sHSPs have partially redundant functions. It will be of interest to confirm these results and test for changes in expression of other chaperones in these CII null plants.

Interestingly, in regular growth conditions, these mutants show no obvious abnormal phenotypes and appear to be just as WT. Differences in seed yield and germination were not closely examined and could be looked at further in the future.

However, it is possible that the plants show reduced thermotolerance in heat stress conditions. Expression patterns along with previous results from heat stress experiments support this. The hypocotyl elongation assay shows that there is not reduced hypocotyl elongation in CII sHSP mutants after heat stress. The root growth assay showed that there might be differences in root growth after heat stress for CII sHSP mutants. However, this should be repeated and examined further as there were different results in the CII sHSP larger deletion mutant and the CII sHSP point mutant.

The initial Hsp17.7-CII double-knock outs retain the introduced CAS9 and guide RNA-containing transgene. To reduce the off-target impacts of Cas9, the mutants were backcrossed to WT. Plants that are homozygous for the mutations for HSP17.6-II and HSP17.7-II without any Cas9 gene for all of the 5 independent lines should be identified for further analysis. Currently, only two of the independent lines were identified (13244-8 and 13251-12). These plants can then be transformed with the complementation vectors to confirm that any phenotypes observed are due to the lack of CII HSPs in the mutant and not to differences in genetic background. Because the sHSPs in the complementation vectors contain affinity tags, the complemented transgenic plants could be used to purify CII interaction partners.

These genetic knockout mutants will give some indication of the importance and phenotype of the CII sHSPs for *A. thaliana in vivo*. This will further elucidate if the CII sHSPs serve a unique function and are necessary despite the presence of other cytosolic CI sHSPs. It may also provide a better understanding of the compensation mechanisms between CI and CII sHSPs. Heat stress experiments at developmental stages could also give some insight into the developmental role of

CII sHSPs. Together with heat stress experiments, this project will give a better understanding of the role of CII sHSPs within a cell.

4. Generating knockout mutants of class I sHSPs

Results and Discussion

Design of gRNAs targeting all six sHSPs

There are no available knockout mutants for all of the *A. thaliana* CI sHSPs. Therefore, to study the role of these proteins, a strategy to disrupt all six CI sHSPs was designed using CRISPR/Cas9 mutagenesis. A similar system to the one used to disrupt the CII sHSP17.7 gene was employed, starting with a vector in which the CAS9 gene is driven by an egg cell specific promoter. The Xing group (Xing, 2014) had a protocol in which 4 gRNAs could be incorporated into a single plasmid. To generate a complete set of CI mutations, two gRNAs needed to be targeted to each CI gene so that there is a greater likelihood for a deletion in the genes. This deletion should then be detectable through PCR genotyping. However, since only 4 gRNAs could be accommodated efficiently and 6 CI sHSP genes needed to each be targeted twice, gRNAs were designed to target multiple genes. This can be achieved by manipulating the ability of gRNAs to bind to off-target genes based on the number of mismatches in the sequence. Using the CRISPRPv2.0 website (http://crispr.hzau.edu.cn/CRISPR2/), gRNAs that target a CI gene and have off-target effects on other CI genes were chosen as gRNAs for the project.

Table 7. gRNAs Targeting CI sHSPs

	gRNA Sequence	On-target gene	On-score	Off-target genes	Off-score
1	GGAGGCGCACGTGTTCAAGGCG	AT1G59860	0.6713	AT1G53540 AT3G46230 AT5G59720	1 0.847 0.672
2	GATCAGTGGAGAGAGACACGTGG	AT2G29500	0.3512	AT1G07400	1
3	GCTGCCGGAGAATGCTAAGATGG	AT1G53540	0.285	AT5G59720	0.744
				AT3G46230	0.632
4	GATAGTGTGCTTAAGATCAGCGG	AT1G59860	0.6246	AT1G07400	0.406
				AT2G29500	0.251
5	GGCGTCAAGAATCCTTCGAACGG	AT3G46230	0.5281	AT1G53540	0.8
				AT5G59720	0.5
6	TCCATTCTCACTCGACGTATGGG	AT1G59860	0.2577	AT2G29500	0.618
				AT1G07400	0.288

				AT3G46230	0.268
7	GAATGACACGTGGCACCGTGTGG	AT2G29500	0.2078	AT1G53540	0.857
				AT3G46230	0.57
				AT5G59720	0.451
8	CATCGTGTGGAGAGGTCGAGCGG	AT1G07400	0.577	AT1G59860	0.605
				AT2G29500	0.403
				***INTRON	0.475

Table 8. gRNA Combinations

Set 1	gRNA 1	gRNA 2	gRNA 3	gRNA 4
Set 2	gRNA 1	gRNA 2	gRNA 7	gRNA 8
Set 3	gRNA 5	gRNA 6	gRNA 3	gRNA 4
Set 4	gRNA 5	gRNA 6	gRNA 7	gRNA 8

Table 9. Primers for Genotyping CI HSP Genes

	Purpose	Sequence (5’ → 3’)	T_A (°C)	Expected Size for WT and **deletion** (bp)
553	Left primer binding to the 5' UTR to genotype AT3G46230 (Hsp17.4-I) with primer 554	AAATACGCCAACGCACATAA	60	702 **525**
554	Right primer binding to the 3' UTR to genotype AT3G46230 (Hsp17.4-I) with primer 553	TGGATCAAAACACTCTTTTATTACGA	60	702 **525**
555	Left primer binding to the 5' UTR to genotype AT1G59860 (Hsp17.6A-I) with primer 556	TCAGCAAACTCACCAGCTTT	59	801 **731**

556	Right primer binding to the 3' UTR to genotype AT1G59860 (Hsp17.6A-I) with primer 555	AACAATAAGATCCATC TCAGTTTCG	59	801 **731**
557	Left primer binding to the 5' UTR to genotype AT2G29500 (Hsp17.B-I) with primer 558	GAAGGCCCATTAAACC CAAC	60	923 **909**
558	Right primer binding to the 3' UTR to genotype AT2G29500 (Hsp17.B-I) with primer 557	CGTCGGTTAAACTTCTT TTGAA	60	923 **909**
559	Left primer binding to the 5' UTR to genotype AT1G53540 (Hsp17.6C) with primer 560	CGAAGATCCCTGATTT TTCAA	59	833 **634**
560	Right primer binding to the 3' UTR to genotype AT1G53540 (Hsp17.6C) with primer 559	AAACAATCCGAGAGGC AGAA	59	833 **634**
561	Left primer binding to the 5' UTR to genotype AT1G07400 (Hsp17.8-I) with primer 562	AAGAAGTTTGGGACCC GTAAA	59	808 **794**
562	Right primer binding to the 3' UTR to genotype AT1G07400 (Hsp17.8-I) with primer 561	TCAGAAAAACAATTGA AACGAGA	59	808 **794**
563	Left primer binding to the 5' UTR to genotype AT5G59720 (Hsp18.1-I) with primer 564	GGATTGCATTTCGGTC TTGT	59	933 **753**
564	Right primer binding to the 3' UTR to genotype AT5G59720 (Hsp18.1-I) with primer 563	CCAACAAAACATTCAC AATGG	59	933 **753**

The gRNAs that comprise Set 1 were chosen as candidates for vector construction. Set 1 uses gRNA 1, gRNA 2, gRNA 3, gRNA 4 to target all the CI genes twice. The location of the gRNA was analyzed to approximate deletion sizes.

In the HSP17.4-I gene (AT3G46230), gRNA 1 binds 190 bp into the coding sequence (off-score: 0.847) and gRNA 3 binds 367 bp into the coding sequence (off-score: 0.632). This is expected to result in a 177 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have a 525 bp fragment due to this 177 bp deletion when using primers 553+534 to genotype the HSP17.4-I gene.

In the HSP17.6A-I gene (AT1G59860), gRNA 1 binds 181 bp into the coding sequence (on-score: 0.6713) and gRNA 4 binds 251 bp into the coding sequence (on-score: 0.6246). This is expected to result in a 70 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have a 731 bp

fragment due to this 70 bp deletion when using primers 555+556 to genotype the HSP17.6A-I gene.

In the HSP17.6B-I gene (AT2G29500), gRNA 2 binds 251 bp into the coding sequence (on-score: 0.3512) and gRNA 4 binds 265 bp into the coding sequence (off-score: 0.251). This is expected to result in a 14 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have an 909 bp fragment due to this 14 bp deletion when using primers 557+558 to genotype the HSP17.6B-I gene.

In the HSP17.6C-I gene (AT1G53540), gRNA 1 binds 173 bp into the coding sequence (off-score: 1) and gRNA 3 binds 373 bp into the coding sequence (off-score: 0.285). This is expected to result in a 200 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have a 634 bp fragment due to this 200 bp deletion when using primers 559+560 to genotype the HSP17.6C-I gene.

In the HSP17.8-I gene (AT1G07400), gRNA 2 binds 260 bp into the coding sequence (off-score: 1) and gRNA 4 binds 274 bp into the coding sequence (off-score: 0.406). This is expected to result in a 14 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have a 794 bp fragment due to this 14 bp deletion when using primers 561+562 to genotype the HSP17.8-I gene.

In the HSP18.1-I gene (AT5G59720), gRNA 1 binds 199 bp into the coding sequence (off-score: 0.672) and gRNA 3 binds 379 bp into the coding sequence (off-score: 0.744). This is expected to result in a 180 bp deletion of the sequence between where the two gRNAs bind. Based on the Table # (CI primers for genotyping), the CI HSP mutants are expected to have a 753 bp fragment due to this 180 bp deletion when using primers 563+564 to genotype the HSP18.1-I gene.

CI CRISPR Vector Cloning and Transformation

The CI CRISPR vector cloning was done by Dr. Patrick Treffon to create p1431. I successfully transformed this plasmid into *A. tumefaciens* for use in plant transformation (**Figure 15**). The vector was used to transform *A. thaliana* wild-type plants, which were subsequently screened on hygromycin plates to obtain transformants.

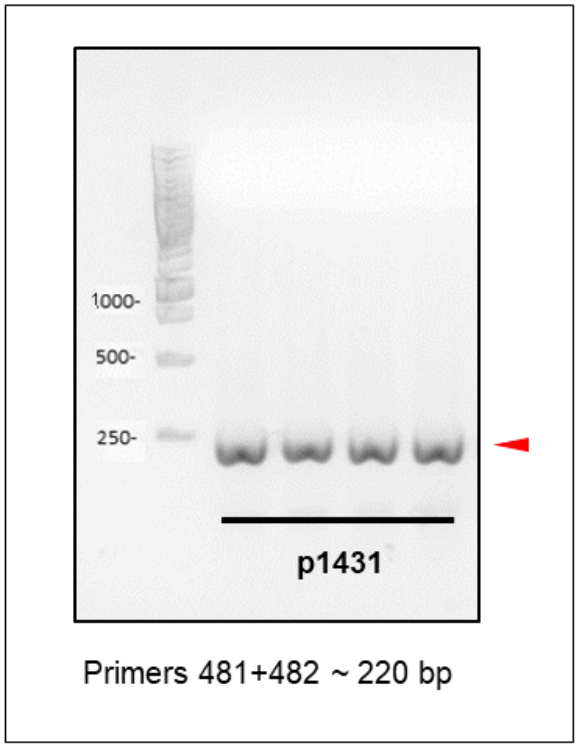

Figure 15. Colony PCR to Confirm Transformation into *A. tumefaciens*

Primers 481+482 bind to the zCas9 gene and amplify a 220 bp product. The 4 lanes are different colonies that were transformed with the p1431 plasmid. All of the colonies had the zCas9 gene and were therefore successfully transformed.

Conclusions and Future Directions

To obtain plants with mutations in all six CI sHSPs, four gRNAs were designed to target all six genes twice. These gRNAs were cloned into a vector containing Cas9 and transformed into WT *A. thaliana* plants. To date 25 plants have been selected as containing the transgene and are being grown on soil. These plants will be tested by PCR to determine if CI sHSP gene mutations can be detected as gene deletions. If there are no deletions in all of the genes, then the genes could be sequenced to determine if there are any point mutations.

Once it is possible to identify one or more plant lines carrying a full set of CI sHSP deletions, expression of CI sHSPs can be tested by immunoblotting to confirm that the mutants are null for CI sHSP protein expression. Then, as has been started for the CII knockout mutants, the plants can then be checked to determine if there is increased expression of other HSPs, which could be compensation for the loss of CI chaperone activity.

These genetic knockout mutants will give some indication of the importance and phenotype of the CI sHSPs for *A. thaliana in vivo*. The mutants can be observed for any differences under regular growth conditions. Additionally, the mutants can be heat stressed and tested for any thermotolerance defects to determine the roles of CI sHSPs in maintaining thermotolerance in plants.

The CI CRISPR vector can also be used to transform the CII dKO mutant to obtain a cytosolic sHSP mutant. By comparing these mutants, it will better show the individual roles of CI and CII sHSPs in plants and also the role of cytosolic sHSPs as a whole.

Complementation vectors with affinity tags can also be constructed to determine if the phenotype is due to the mutation and not any other factors. These tags can also be used for protein purification.

References

Alberts B., Bray D., Hopkin K., Johnson A., Lewis J., Raff M., Roberts K. & Walter P. (2014) Essential Cell Biology (pp. 123-140)

Ballard, K.N. (2018) Examining sHSP-Substrate Capture and Chaperone Network Coordination Through Crosslinking. PhD Thesis. University of Massachusetts Amherst.

Basha, E., Lee, G.J., Demeler, B., and Vierling, E. (2004). Chaperone activity of cytosolic small heat shock proteins from wheat. Eur J Biochem *271*, 1426–1436.

Basha, E., Jones, C., Wysocki, V., and Vierling, E. (2010). Mechanistic Differences between Two Conserved Classes of Small Heat Shock Proteins Found in the Plant Cytosol. J. Biol. Chem. *285*, 11489–11497.

Basha, E., Friedrich, K.L., and Vierling, E. (2006). The N-terminal Arm of Small Heat Shock Proteins Is Important for Both Chaperone Activity and Substrate Specificity. J. Biol. Chem. *281*, 39943–39952.

Delbecq SP, Klevit RE (2013). One Size Does Not Fit All: The Oligomeric States of alphaB crystallin. FEBS Lett 587, 1073–1080.

Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., et al. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol *34*, 184–191.

Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B.L., Xavier, R.J., and Root, D.E. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation. Nat Biotechnol *32*, 1262–1267.

Giese, K.C., and Vierling, E. (2004). Mutants in a Small Heat Shock Protein That Affect the Oligomeric State: Analysis and Allele-Specific Suppression. J. Biol. Chem. *279*, 32674–32683.

Götzke, H., Kilisch, M., Martínez-Carranza, M., Sograte-Idrissi, S., Rajavel, A., Schlichthaerle, T., Engels, N., Jungmann, R., Stenmark, P., Opazo, F., et al. (2019). The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. Nat Commun *10*, 4403.

Guo, L.-M., Li, J., He, J., Liu, H., and Zhang, H.-M. (2020). A class I cytosolic HSP20 of rice enhances heat and salt tolerance in different organisms. Sci Rep *10*, 1383.

Guo, M., Liu, J.-H., Ma, X., Luo, D.-X., Gong, Z.-H., and Lu, M.-H. (2016). The Plant Heat Stress Transcription Factors (HSFs): Structure, Regulation, and Function in Response to Abiotic Stresses. Front. Plant Sci. *7*.

Haslbeck, M., and Vierling, E. (2015). A First Line of Stress Defense: Small Heat Shock Proteins and Their Function in Protein Homeostasis. Journal of Molecular Biology *427*, 1537–1548.

Helm, K.W., Lee, G.J., and Vierling, E. (1997). Expression and Native Structure of Cytosolic Class II Small Heat-Shock Proteins. Plant Physiol. *114*, 1477–1485.

Hofmann, S., Kedersha, N., Anderson, P., and Ivanov, P. (2021). Molecular mechanisms of stress granule assembly and disassembly. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1868*, 118876.

Hong, S.-W., Lee, U., and Vierling, E. (2003). Arabidopsis hot Mutants Define Multiple Functions Required for Acclimation to High Temperatures. Plant Physiology 132, 757–767.

Li, C., Hao, M., Wang, W., Wang, H., Chen, F., Chu, W., Zhang, B., Mei, D., Cheng, H., and Hu, Q. (2018). An Efficient CRISPR/Cas9 Platform for Rapidly Generating Simultaneous Mutagenesis of Multiple Gene Homoeologs in Allotetraploid Oilseed Rape. Front. Plant Sci. *9*, 442.

Mayer, M.P., and Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. CMLS, Cell. Mol. Life Sci. *62*, 670–684.

McLoughlin, F., Basha, E., Fowler M.E., Kim M., Bordowitz J., Kaityar-Agarwal S., & Vierling, E. (2016). Class I and II Small Heat Shock Proteins Together with HSP101 Protect Protein Translation Factors During Heat Stress. Plant Physiol 172, 1221-1236.

McQuillan, A. (2017) Genetic Analysis of the Function of Specific Small Heat Shock Proteins in Plants

Miroshnichenko, S., Tripp, J., Nieden, U. zur, Neumann, D., Conrad, U., and Manteuffel, R. (2004). Immunomodulation of function of small heat shock proteins prevents their assembly into heat stress granules and results in cell death at sublethal temperatures: Immunomodulation of small heat shock proteins. The Plant Journal *41*, 269–281.

Mogk, A., Schlieker, C., Friedrich, K.L., Schönfeld, H.-J., Vierling, E., and Bukau, B. (2003). Refolding of Substrates Bound to Small Hsps Relies on a Disaggregation Reaction Mediated Most Efficiently by ClpB/DnaK. J. Biol. Chem. 278, 31033–31042.

Nagao RT, Czarnecka E, Gurley WB, Schoffl F, Key JL (1985). Genes for low-molecular-weight heat shock proteins of soybeans: sequence analysis of a multigene family. Mol Cell Biol 5, 3417–3428

Neef, D.W., Jaeger, A.M., Gomez-Pastor, R., Willmund, F., Frydman, J., and Thiele, D.J. (2014). A Direct Regulatory Interaction between Chaperonin TRiC and Stress-Responsive Transcription Factor HSF1. Cell Reports *9*, 955–966.

Santhanagopalan, I., Basha, E., Ballard, K. N., Bopp, N. E., & Vierling, E. (2015). Model Chaperones: Small Heat Shock Proteins from Plants. The Big Book on Small Heat Shock Proteins (pp. 119–153)

Santhanagopalan, I., Degiacomi, M.T., Shepherd, D.A., Hochberg, G.K.A., Benesch, J.L.P., and Vierling, E. (2018). It takes a dimer to tango: Oligomeric small heat shock proteins dissociate to capture substrate. J. Biol. Chem. *293*, 19511–19521.

Sarkar, N.K., Kotak, S., Agarwal, M., Kim, Y.-K., and Grover, A. (2020). Silencing of class I small heat shock proteins affects seed-related attributes and thermotolerance in rice seedlings. Planta *251*, 26.

Virant, D., Traenkle, B., Maier, J., Kaiser, P.D., Bodenhöfer, M., Schmees, C., Vojnovic, I., Pisak-Lukáts, B., Endesfelder, U., and Rothbauer, U. (2018). A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. Nat Commun *9*, 930.

Waters, E.R., Aevermann, B.D., and Sanders-Reed, Z. (2008). Comparative analysis of the small heat shock proteins in three angiosperm genomes identifies new subfamilies and reveals diverse evolutionary patterns. Cell Stress and Chaperones 13, 127–142.

Waters, E.R., Lee, G.J., and Vierling, E. (1996). Evolution, structure, and function of the small heat shock proteins in plants. J Exp Bot *47*, 325–338.

Wehmeyer, N., Hernandez', L.D., and Vierling, E. (1996). Synthesis of Small Heat-Shock Proteins 1s Part of the Developmental Program of l a t e Seed Maturation'. 11.

Miroshnichenko, S., Tripp, J., Nieden, U. zur, Neumann, D., Conrad, U., and Manteuffel, R. (2004). Immunomodulation of function of small heat shock proteins prevents their assembly into heat stress granules and results in cell death at sublethal temperatures: Immunomodulation of small heat shock proteins. The Plant Journal *41*, 269–281.

Nagao RT, Czarnecka E, Gurley WB, Schoffl F, Key JL (1985). Genes for low-molecular-weight heat shock proteins of soybeans: sequence analysis of a multigene family. Mol Cell Biol 5, 3417–3428

Neef, D.W., Jaeger, A.M., Gomez-Pastor, R., Willmund, F., Frydman, J., and Thiele, D.J. (2014). A Direct Regulatory Interaction between Chaperonin TRiC and Stress-Responsive Transcription Factor HSF1. Cell Reports *9*, 955–966.

Santhanagopalan, I., Basha, E., Ballard, K. N., Bopp, N. E., & Vierling, E. (2015). Model Chaperones: Small Heat Shock Proteins from Plants. The Big Book on Small Heat Shock Proteins (pp. 119–153)

Santhanagopalan, I., Degiacomi, M.T., Shepherd, D.A., Hochberg, G.K.A., Benesch, J.L.P., and Vierling, E. (2018). It takes a dimer to tango: Oligomeric small heat shock proteins dissociate to capture substrate. J. Biol. Chem. *293*, 19511–19521.

Sarkar, N.K., Kotak, S., Agarwal, M., Kim, Y.-K., and Grover, A. (2020). Silencing of class I small heat shock proteins affects seed-related attributes and thermotolerance in rice seedlings. Planta *251*, 26.

Vendredy, L., Adriaenssens, E., and Timmerman, V. (2020). Small heat shock proteins in neurodegenerative diseases. Cell Stress and Chaperones *25*, 679–699.

Waters, E.R., Lee, G.J., and Vierling, E. (1996). Evolution, structure, and function of the small heat shock proteins in plants. J Exp Bot *47*, 325–338.

Wehmeyer, N., Hernandez', L.D., and Vierling, E. (1996). Synthesis of Small Heat-Shock Proteins 1s Part of the Developmental Program of l a t e Seed Maturation'. 11.

Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Wang, X.-C., and Chen, Q.-J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14, 327.

Xiong, J., Li, Y., Tan, X., and Fu, L. (2020). Small Heat Shock Proteins in Cancers: Functions and Therapeutic Potential for Cancer Therapy. IJMS *21*, 6611.

Xu, H. (2018). Cochaperones enable Hsp70 to use ATP energy to stabilize native proteins out of the folding equilibrium. Sci Rep *8*, 13213.

Zhang, L., Hu, W., Gao, Y., Pan, H., and Zhang, Q. (2018). A cytosolic class II small heat shock protein, PfHSP17.2, confers resistance to heat, cold, and salt stresses in transgenic Arabidopsis. Genet. Mol. Biol. *41*, 649–660.

Zhou, Y., Chen, H., Chu, P., Li, Y., Tan, B., Ding, Y., Tsang, E.W.T., Jiang, L., Wu, K., and Huang, S. (2012). NnHSP17.5, a cytosolic class II small heat shock protein gene from Nelumbo nucifera, contributes to seed germination vigor and seedling thermotolerance in transgenic Arabidopsis. Plant Cell Rep *31*, 379–389.

Appendix

Final Table of Plasmids Designed/Cloned/Transformed

	Description
p1412	pREDSEED-HSP17.6-FLAG/HSP17.7-HA
p1413	pREDSEED-HSP17.6-FLAG
p1414	pREDSEED-HSP17.7-HA
p1415	pREDSEED-HSP17.6-BC2t/HSP17.7-ALFA
p1429	pHEE401 - Set 1 gRNAs
p1430	pHEE401 - Set 2 gRNAs
p1431	pHEE401- Set 1 and Set 2 gRNAs