Class I (CI) Small Heat Shock Proteins (sHSPs)

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Introduction

Small heat shock proteins (sHSPs) assist with the formation and maintenance of the 3D structure of other proteins. In plants, sHSPs are highly expressed at elevated temperatures. Our goal is to understand the function of sHSPs in the response of plants to high temperature stress. These proteins may be increasingly important for plant health in relation to climate change. This research focuses on the six cytosolic Class I (CI) sHSPs in *Arabidopsis thaliana*, which are highly expressed in response to heat stress. In order to understand the function of the six CI sHSPs, Hsp17.4-I, 17.6A-I, 17.6B-I, 17.6C-I, 17.8-I, and 18.1-I (Figure 1), we are working to generate null mutants in the corresponding genes using CRISPR-Cas methods (Figure 2). Previous work had already identified a deletion mutant of 17.6C-I, and has transformed wild type *A. thaliana* plants with guide RNAs to generate mutant lines of 17.4-I 17.6A-I, 17.6B-I, 17.8-I and 18.1-I. Here we report on progress toward establishing single mutants of each of these CI sHSP genes. Work is also in progress to analyze mutants of the Class II (CII) sHSPs genes. Ultimately we will test the phenotype of mutant plants to determine the role of these sHSPs during stress and normal growth and development.

Specific Project Goals

➢ To identify an HSP17.6-CI mutant plant line, which through segregation, no longer carries the CRISPR/Cas vector DNA.

➢ To identify CRISPR large deletion or indel mutants of the other 5 CI sHSP genes.

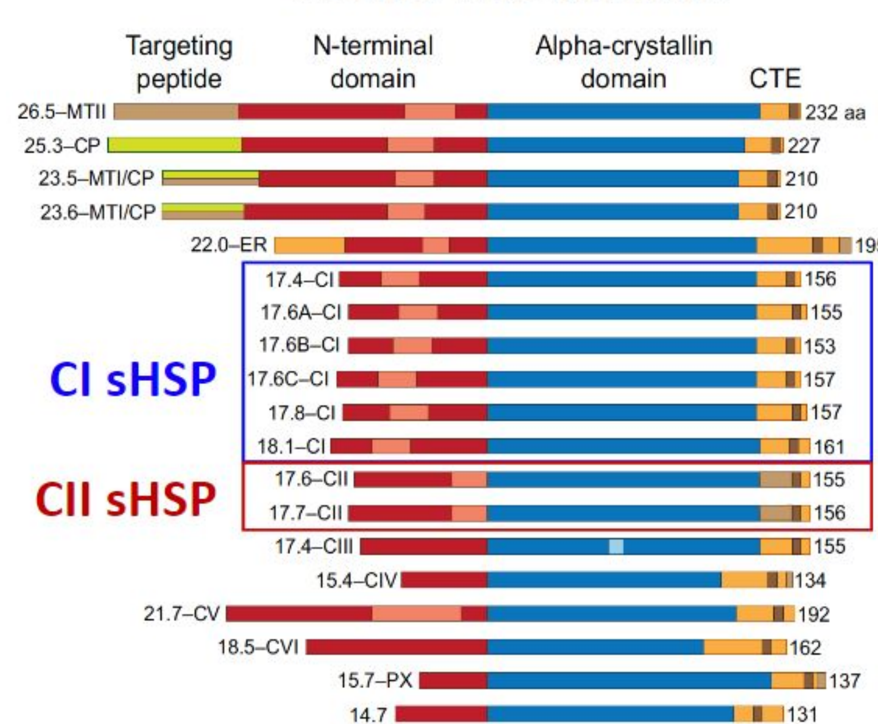

Figure 1. Domain structure of the 19 sHSPs in *Arabidopsis thaliana*. There are 6 CI and 2 CII sHSP genes.

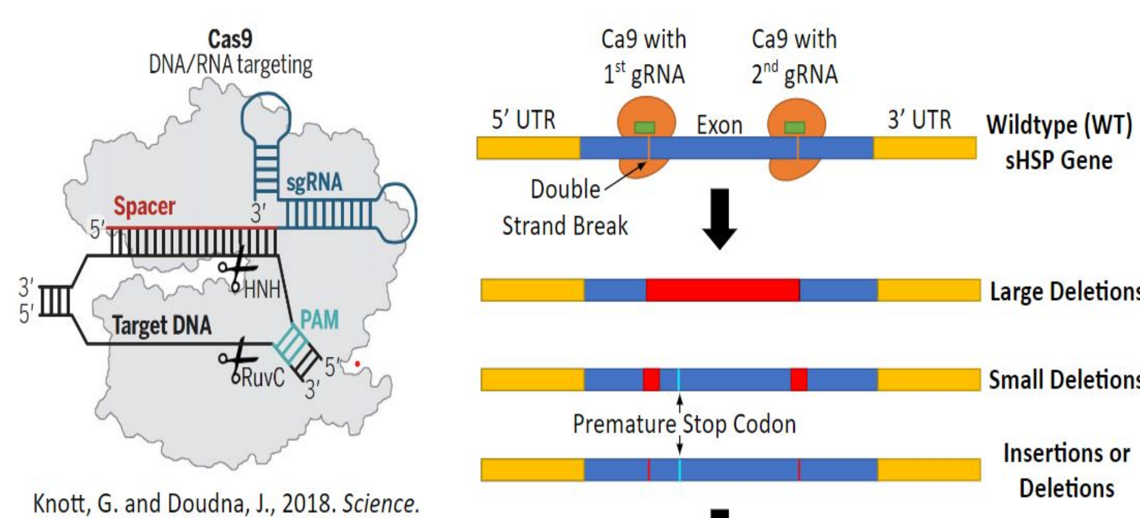

Figure 2. CRISPR-Cas9 Can Generate Mutations At Specific Sequences.

Methods

Testing for the presence of the CRISPR/Cas9 DNA in transgenic 17.6-I plants: Two approaches were used. Seeds were plated on media with or without hygromycin, the selectable marker on the CRISPR/Cas vector, to identify lines no longer carrying hygromycin resistance (Tables 1 & 2). As an additional test, DNA was extracted from individual seedlings of each transgenic line, and polymerase chain reaction (PCR) was used to amplify the hygromycin gene, confirming its presence or absence (FIgure 3).

Identifying CRISPR/Cas9 mutations in transgenic plants: Twelve plants of each sHSP transgenic line were grown to extract DNA for PCR genotyping. Specific primers were designed (Table 3) to PCR amplify DNA at each different mutation site, and results analyzed by agarose gel electrophoresis (Figure 5). As required, amplified DNA fragments were sent out for DNA sequencing to identify indel mutations that cannot be detected by electrophoresis.

Results

➔ 17.6C-I: Establishing a CRISPR/Cas-free line

Hygromycin plate screening and Hygromycin PCR Genotyping

Vierling Lab plant line # for 17.6C-I mutants: 13466-1 #1 & #9 - 176 bp deletion; 13466-3 #2 ,#3 #5, #8, #12 & #15 - 177 bp deletion; 13466-4, 176 bp deletion.

Table 1

Hygromycin Plate				Control Plate			
Plant line	Growth	Not growth	Growth Percentage	Plant line	Growth	Not growth	Growth Percentage
WT	0	100	0	WT	8	92	8
13466-1 #1	6	94	6	13466-1 #1	3	97	3
13466-1 #9	0	100	0	13466-1 #9	5	95	5
13466-3 #2	61	39	61	13466-3 #2	66	34	66
13466-3 #3	84	16	84	13466-3 #3	88	12	88
13466-3 #5	77	23	77	13466-3 #5	88	12	88
13466-3 #8	63	37	63	13466-3 #8	73	27	73
13466-3 #12	73	27	73	13466-3 #12	83	17	83
13466-3 #15	68	32	68	13466-3 #15	77	23	77
13466 4	94	6	94	13466 4	99	1	99

Table 1: Test #1 of growth on hygromycin of 17.6C-I mutants. 100 seeds per line were grown on plates with or w/o (Control) hygromycin.

Table 2

Hygromycin rescreen plate				Control Plate			
Plant line	Growth	Not growth	Growth percentage	Plant line	Growth	Not growth	Growth Percentage
WT	186	14	93	WT	198	2	99
13466-4	176	24	88	13466-4	195	5	97.5
13466-4	177	23	88.5	13466-1-9	5	195	2.5
13466-1-9	0	200	0				
13466-1-9	0	200	0				

Table 2: Retest of Lines 13466-4 and 13466-1 #9. 200 seeds per line. Line 13466-1-9 plant #1 does not grow on hygromycin and should be CAS free. Liine 13345-4 appears heterozygous for hygromycin resistance.

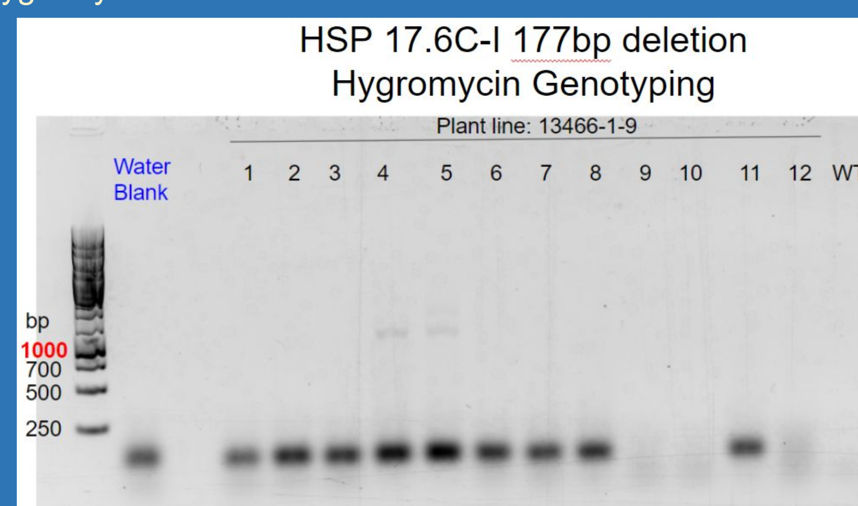

Figure 3: PCR amplification of the Hygromycin gene from 12 individual F3 13466-1-9 plants using primers 766+767 (Table 3) , expected product size is 972. This line lacks the hygromycin gene, as predicted from the growth data in Tables 1 & 2.

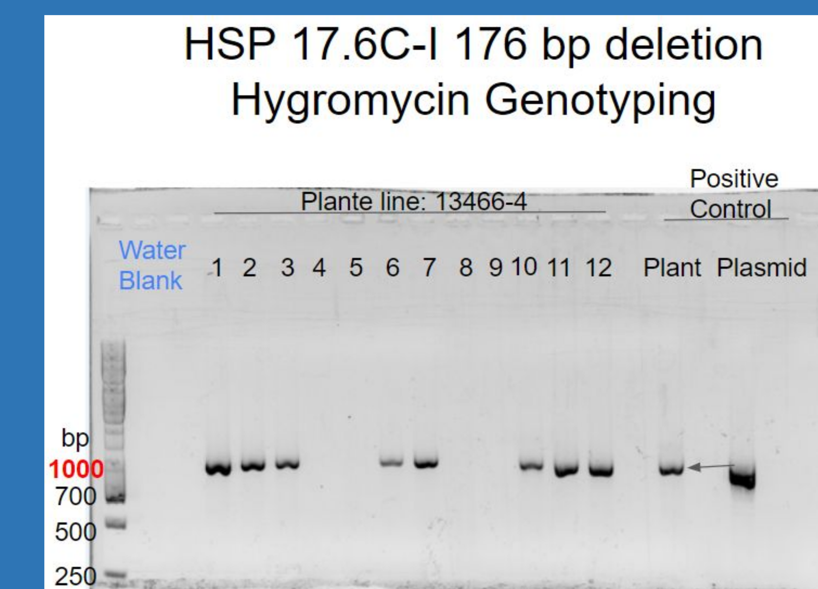

Figure 4: Performed as in Figure 3, with plant line 13346 4. Expected product size is 972, plants numbered 4, 5, 8, 9 were confirmed to be Cas-free in an another PCR reaction.

➔ Identifying new sHSP-CI mutants: 17.4-I,17.6A-I, 17.6B-I, 17.8-I and 18.1-I

HSP17.4-I Genotyping, F1 #1-5

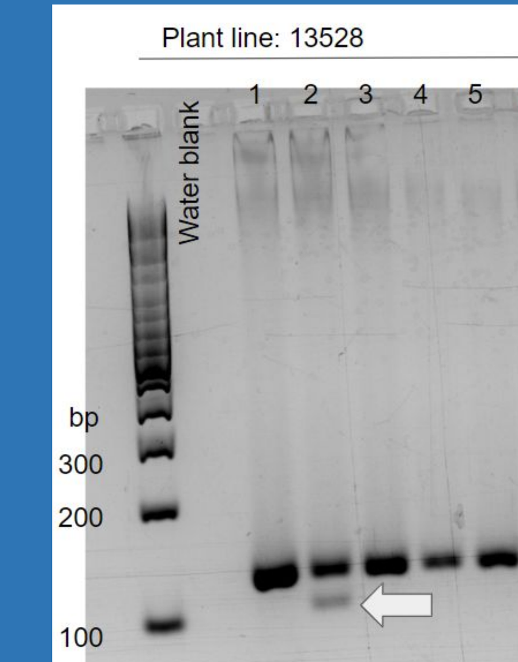

Figure 5: Genotyping the 17.4-I gene, line # 13528 with primers 894+895 (Table 3). The expected product size in a mutant is 105bp (arrow). Plant #2 appears heterozygous for a deletion mutation.

HSP17.8-I Genotyping, F1 #1-7

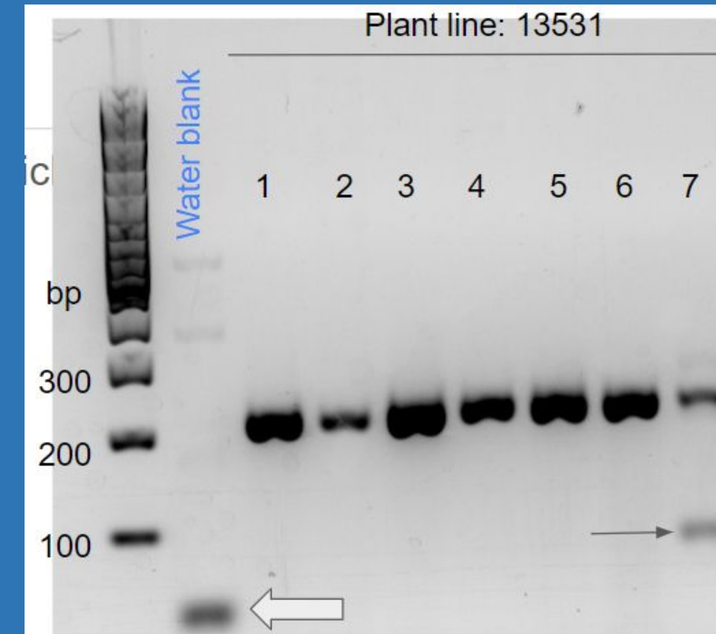

Figure 6: Genotyping the 17.8-I gene, Line # 13531, with primers 900 + 901. The expected product size for mutant is 54bp, WT is 199bp. The small arrow points to a band with the expected size for a deletion mutation. The big arrow indicate a non-specific PCR amplification. Plant #7 appears heterozygous for a deletion mutation.

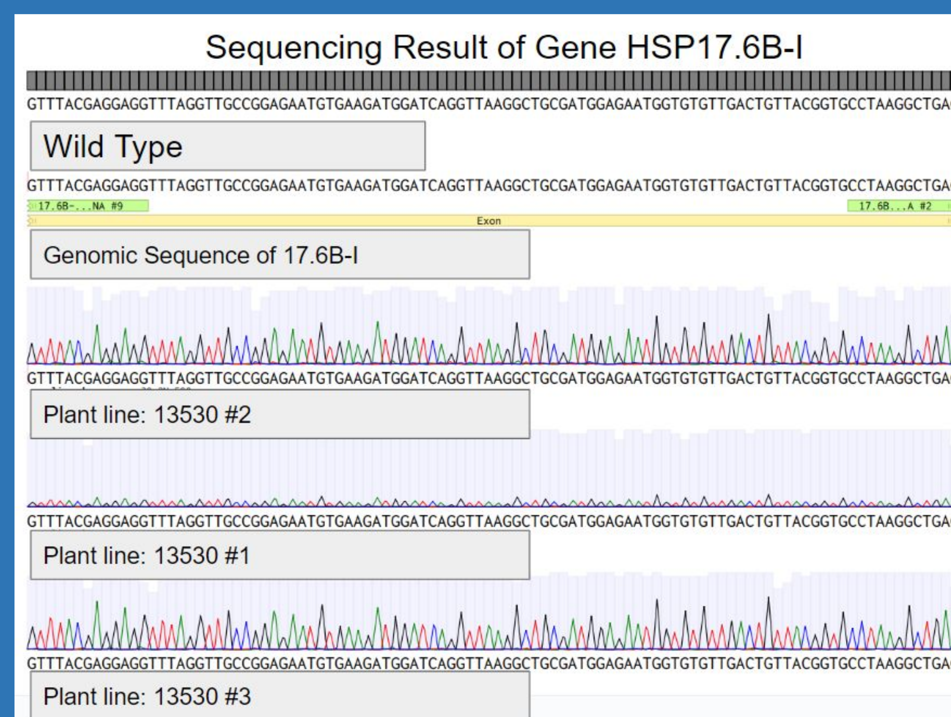

Figure 7: Sequencing of 17.6B-I gene mutation. The alignment used WT as a template. The green bars in the Genomic Sequence show position of the CRISPR guide RNAs where mutation expected.

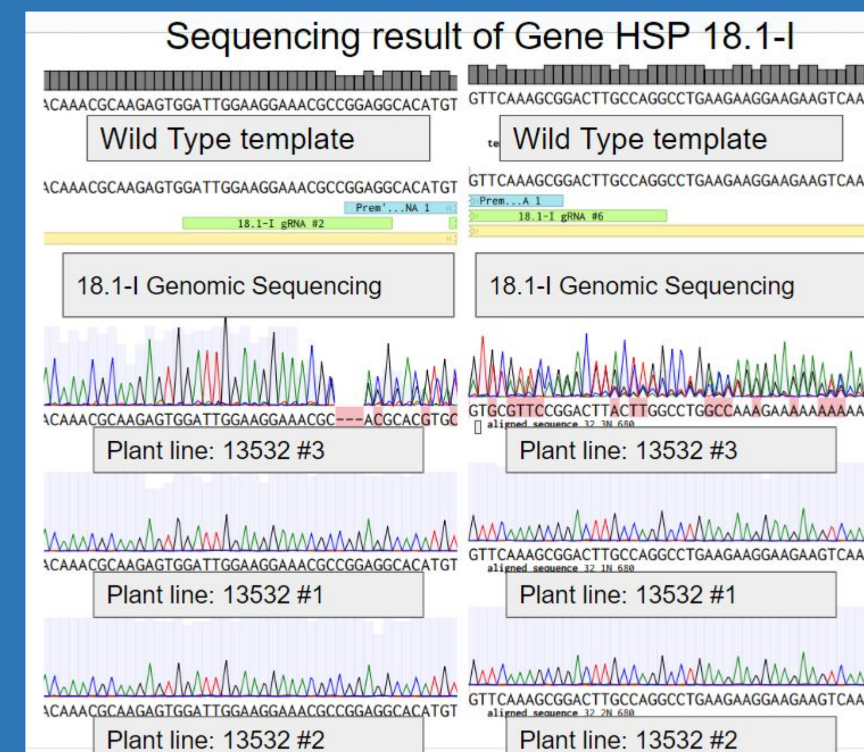

Figure 8: Sequencing result of 18.1-I, using WT as the alignment template. The red block of nucleotides do not align with the template, "----" indicates insertion or deletion. Green bars in the Genomic Sequence as in Figure 7.

Conclusions

➔ 17.6C-I: Establishing a CRISPR/Cas-free line

- ◆ The T3 generation line 13466-1-9 plant #1, with a 176 deletion is hygromycin free and therefore is a CRISPR/Cas-free line that can be used for future analysis and transformation.
- ◆ The T2 generation line 13345-4 plants #4 5 8 9, with a 177 bp mutation are CRISPR/Cas-free.

➔ Identifying new sHSP-CI mutants

- ◆ For the 17.4-I and 17.8-I, genes at least one plant that is heterozygous for a deletion mutation has been identified.
- ◆ Sequencing results of line 18.1-I showed that plant #3 has a single base insertion and a small deletion of 3 bases.
- ◆ So far no mutations have been found in 17.6A-I or 17.6B-I.

➔ In total mutations have been identified in 4 of the six CI sHSP genes.

Future Directions

➔ **17.6C-I gene mutation:** With the established CRISPR/Cas-free line, we will confirm the exact mutation site by amplifying the gene by PCR and sequencing. Once the mutation has been confirmed, this plant will be backcrossed with WTfor future phenotypic analysis.

➔ **Identifying new sHSP-CI mutants:** For lines 17.8-I and 17.4-I with heterozygous mutations, we will harvest T2 seed and screen for homozygous mutants. For lines 17.6A-I and 17.6B-I, we will screen more T1 transformants as well as T2 plants, because the CRISPR/Cas machinery could work in subsequent generations.

➔ **Creating a Mutant lacking all 6 CI sHSPs:** Established CRISPR-free CI mutant lines will be crossed together and/or transformed with CRISPR constructs to add mutations in other CI genes.

Table 3 - Primers to detect mutations in sHSP genes.

Primer Number	Forward/ Reverse	Sequence	HSP Gene amplified (AGI #)/ (CRISPR plasmid)
894	F	GCTAAAGTGGACTGGAGGGA	17.4-I (AT3G46230)/(p1456)
895	R	GCTCCTCTCACCGCTTATCT	"
896	F	AGCTTCTTCGGCAACAACAG	17.6A-I (AT1G59860)/(p1457)
897	R	GTGTCTCTCTCCGCTGATCT	"
898	F	GTGGAGAGAGACACGTGGAG	17.6B-I(AT2G29500)/(p1458)
899	R	AACACAAGCAACTCCGATGA	"
900	F	TGGAAAGAGACGGCGGAG	17.8-I(At1g07400) /p1460)
901	R	CCGGCAGCTTAAACTTCCTC	"
902	F	GTGATGTGGCAGCGTTTACA	18.1-I(At5g59720)/(p1461)
903	R	CCTCTCTCCGCTAATCTGCA	"

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McLaughlin, D. (2023) Establishment of Cytosolic Small Heat Shock Protein Knockout Lines in *Arabidopsis thaliana.* UMass, Senior Honors Thesis.

Vierling Lab Data Base.

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