Arabidopsis HSP21 and MsrB1/MsrB2 in plant stress tolerance.

By

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Overall Research Question:

Does HSP21 have a unique role in protecting the viability of the organism under heat stress? How do the proteins MsrB1 and MsrB2 relate to HSP21 and what is the mechanism of their interaction that may lead to greater survival under both oxidative and heat stress conditions?

Specific Aims

Aim 1: Do *Arabidopsis* plants that lack HSP21 show a significant phenotype?
Western analysis and genotyping has shown that plants with homozygous splice site mutation, line 10-4, express little or no HSP21 after heat stress. In order to determine the phenotype of these plants, the mutation will be backcrossed to separate it from other mutations, and western analysis will be used to confirm that line10-4 is a null mutation. Using backcrossed plants, several assays will be performed to examine growth and heat tolerance.

Aim 2: What role do the MsrB1 and MsrB2 proteins have in protecting HSP21 under heat or oxidative stress?
Preliminary western analysis has shown that MsrB1/MsrB2 knockout plants accumulate less HSP21 under heat stress. The effect of the MsrB1/MsrB2 knockout on HSP21 during heat and oxidative stress will be explored. It has been shown that MsrB1 and MsrB2 are localized to the cholorplast like HSP21 and may play a role in protecting the chloroplasts from radical oxygen species by reducing oxidized methionines. Lack of MsrB1 and MsrB2 under heat stress may lead to irreversible oxidation of HSP21 methionines, leading to a non-functional protein. Viability of the MsrB1/B2 knockout plants will be explored under stress conditions.

***Arabidopsis* HSP21 and MsrB1/MsrB2 in plant stress tolerance.**

Abstract:

Small heat shock proteins (sHSPs) are molecular chaperones that assist in the protection of substrates from irreversible aggregation when organisms experience high temperature stress. Plant species such as the model organism *Arabidopsis thaliana*, have evolved this mechanism to adapt to varying temperatures and to acquire thermotolerance, both of which are essential to survival of the organism under changing conditions. There are a total of 19 sHSPs in *Arabidopsis thaliana* which are categorized by their amino acid sequence, as well as the compartment to which they are localized within the cell. Hsp21 is the sole sHSP localized to the chloroplast and is therefore particularly interesting and amenable to genetics studies of sHSP function in the chloroplast. Hsp21 also has a unique Met-rich α-helix, known as a "met bristle" which is thought to be important for Hsp21 function, particularly during oxidative stress. Plants with a homozygous mutation at the 3' splice site of the only intron in the Hsp21 gene were confirmed to have reduced or no Hsp21 expression. These mutant plants will allow study of the importance of chloroplast-localized Hsp21 function in plants. The splice site mutant has been backcrossed to wildtype as the first step in securing homozygous mutants free from background mutations. Recent purification of HSP21 will also allow for crystallization and structure identification.

Also, the structural characteristics of conserved Met residues have led us to investigate methionine sulfoxide reductase proteins (Msr) that normally play a role in oxidative stress. Two types of Msr proteins have been identified and named MsrA and MsrB proteins. In *Arabidopsis*, there are 14 total Msr proteins (5 MsrA and 9 MsrB) that

are found in the cytosol, chloroplasts and secretory pathway. Only two, MsrB1 and MsrB2, are localized to the chloroplast. These two proteins, in conjunction with Hsp21, will be experimented for correlations in dealing with both oxidative and heat stress. Currently, a double knockout MsrB1/ MsrB2 mutant, as well as each single mutant has been assayed via heat stressed hypocotyl elongation and 10 day light treatments. Preliminary data has shown that the MsrB1/B2 double knockout and MsrB1 have decreased growth during heat stress.

Introduction:

Organisms have evolved a mechanism for protecting proteins from denaturation and irreversible aggregation, which can occur at high temperatures. The mechanism involved makes use of heat shock proteins (Hsps) that play various roles in the cell, but a major role is preventing cell death due to stress. Small heat shock proteins (sHsps) were identified by their synthesis during heat stress, but they are also induced during development and found

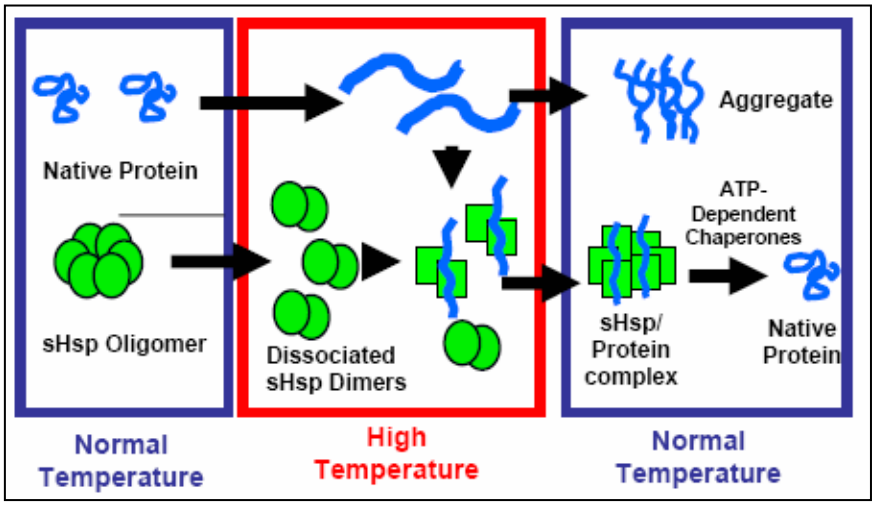

Figure 1: Model of sHsp Function as Chaperones.

sHsps dissociate from the oligomeric form into dimers upon high temperature and bind to denatured substrate proteins. The complex protects proteins until return to normal temperatures, and when the denatured proteins are refolded by ATP-dependent chaperones.

normally in some tissues. Moderately high, non-lethal temperatures can induce sHsps and allow for us to study their expression *in vivo* .

Although the mechanisms of Hsps are complex and not fully understood, most function as chaperones *in vitro* to protect against irreversible protein aggregation. It is currently proposed that sHsps function as chaperones to aid in the protection of proteins during heat stress. Figure 1 shows the current model by which sHsps are thought to protect substrate proteins from irreversible aggregation after heat stress and to facilitate their return to the native state by ATP-dependent chaperones. Substrate binding is thought to be initiated by hydrophobic amino acid residues exposed on denatured proteins. Upon return from heat stress, these proteins can be released and refolded to their native conformation by other chaperones. (Siddique et al., 2008). Consequently, protein folding patterns and structure are very important to understanding sHsp function. Figure 2.illustrates the oligomeric structure of Hsp16.9B from *Triticum aestivum* and shows that the native structure of one plant sHsp is a donut-shaped dodecamer (van Montfort, 2001)

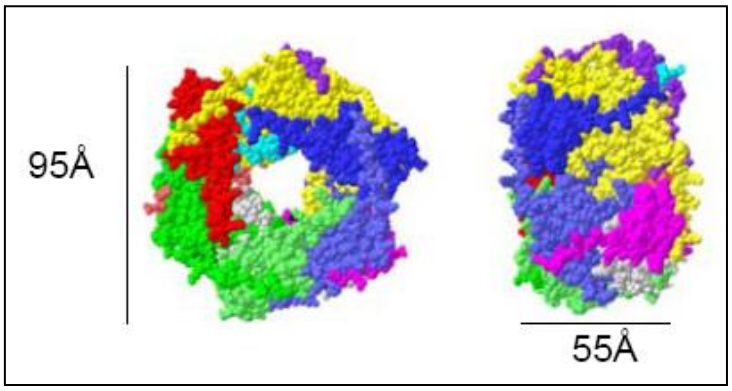

Figure 2: Crystal Structure of TaHsp16.9

The crystal structure of Hsp16.9 from *Triticum aestivum*, showing the dodecameric oligomeric structure (van Monfort et al 2001)

sHsps range in size from 15 to 30 kDa and show a conserved carboxyl-terminal heat shock domain (approximately 90 amino acids) that is found in all other eukaryotic sHsps and is also noted to belong to a family of proteins with alpha-crystallin domains

(Chen et al., 1994). The genome of the model organism *Arabidopsis thaliana* has revealed 19 sHSP ORFs which were separated into 7 subfamilies, Class I (CI), CII, CIII, Endoplasmic Reticulum (ER), Plastids (P), Mitochondria (M), and peroxisomes (Po), but newly published work indicates 5 more subfamilies (Siddique et al., 2008) . Within the seven classes listed, three classes (CI, CII, and CIII) are localized to cytoplasm and nucleus and localization of the other sHsps is indicated by their class name. The 19 sHsps from Arabidopsis are listed in Table 1, including their accession numbers, compartmentalization and size based on molecular mass, given in kilo Daltons and listed within its name. The classification via compartmentalization is also reflected in specific sequence motifs in the N-terminal domain and C-terminal extensions (Siddique et al., 2008). Within this study, the focus is on AtHsp25.3-P (Hsp21), the only *Arabidopsis* sHsp localized to the chloroplast. As the mature form of Hsp25.3-P is generated from its precursor upon import into the organelle and is only 21 kDa, this sHsp is also commonly

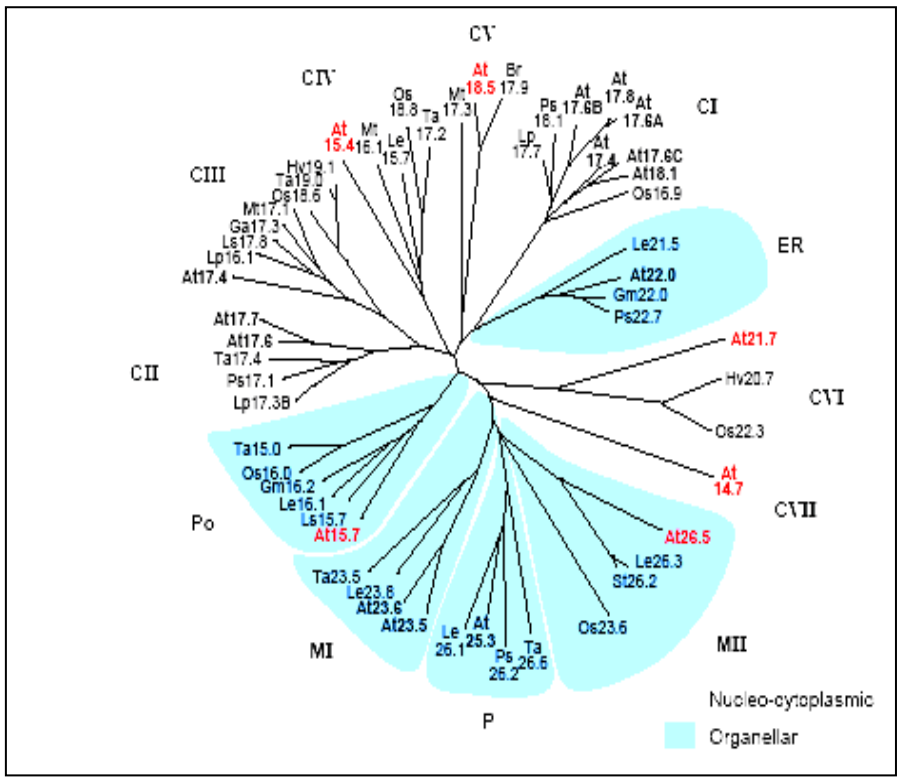

Figure 3: Phylogenetic tree showing the 12 classes of Hsps in *Arabidopsis* and other organisms by evolutionary relationships. (Siddique, et al, 2008)

referred to as Hsp21, which is the terminology used throughout the remainder of this thesis.

Hsp21 is of particular interest for a variety of reasons. First, it is predicted that a specific motif in the N-terminal arm of Hsp21 forms an amphipathic α-helix with a region of conserved methionines. Secondary structure predicts high conservation of methionines on the hydrophobic side and 100% conservation on the hydrophilic face, termed "methionine bristles" (Chen et al., 1991). From this unique characteristic, Hsp21 is assumed to play an important role in substrate protection and an even more vital role as the sole sHsp in the chloroplast. The structure of Hsp21 with the proposed methionine bristle could allow efficiency in binding to multiple substrates of various compositions, making the protein's function multifaceted (Raschke et al., 1988)

Gene	Accession #	Localization
Hsp25.3(21)-P	At4g27670	Chloroplast
Hsp26.5-M	At1g52560	Mitochondrion
Hsp23.6-M	At4g25200	Mitochondrion
Hsp23.5-M	At5g51440	Mitochondrion
Hsp14.7-P(r)	At5g47600	Plastid?
Hsp21.7-CI(r)	At5g54660	Cytosol
Hsp17.6B-CI	At2g29500	Cytosol
Hsp17.8-CI	At1g07400	Cytosol
Hsp17.6A-CI	At1g59860	Cytosol
Hsp18.1-CI	At5g59720	Cytosol
Hsp17.4-CI	At3g46230	Cytosol
Hsp17.6C-CI	At1g53540	Cytosol
Hsp18.5-CI(r)	At2g19310	Cytosol
Hsp22.0-ER	At4g10250	ER
Hsp15.7-Po	At5g37670	Peroxisome
Hsp17.6-CII	At5g12020	Cytosol
Hsp17.7-CII	At5g12030	Cytosol
Hsp17.4-CIII	At1g54050	Nucleus
Hsp15.4-CI(r)	At4g21870	Cytosol

Table 1: List of Hsp Proteins in *Arabidopsis.* (Corrigan, thesis 2006)

The family of sHsps, including Hsp21, can be induced by a variety of other stresses in addition to heat stress. Cold, drought, salinity, and developmental processes are just some examples where sHsps are induced and may function to protect cells. Some studies have shown that Hsp21 protects the thermolabile photosystem II during heat stress in isolated chloroplasts (Heckathorn et al., 1998). Transgenic *Arabidopsis* constitutively expressing Hsp21 have shown an increased tolerance to heat and high-light stresses (a combination that induces oxidative stress) (Harndahl et al., 1999). From these

results, other groups have predicted that Hsp21 acts as an antioxidant under oxidative stress (Levine et al., 1996). In tomato (*Lycopersicon esculentum)*, transgenic plants constitutively expressing Hsp21 protected photosystem II from temperature-dependent oxidative stress, as well as playing a role in fruit maturation by promoting accumulation of carotenoids (Neta-Sharir et al., 2005). The mechanism by which Hsp 21 has these effects is poorly understood.

Hsp21 may prevent aggregation and facilitate refolding of components of photosystem II as is predicted in other papers (Neta-Sharis, Weiss 2005). Also, the Met-rich domain of the chloroplast sHsp could play a role as an antioxidant to prevent damage from reactive oxygen species (ROS) (Levine et al., 1996). sHsps also have been proposed to protect cellular proteins through a mechanism involving ROS scavenging, similar to that of antioxidants, and it has been suggested that the mechanism involves scavenging by the Met residues (possibly via the Met bristle) (Harndahl et al., 2001). Under oxidative stress conditions, the Met residues can oxidize to Met sulfoxide and thus shield other oxidation-sensitive proteins (Levine et al., 1996). Sulfoxidation of the Met residues causes conformational changes and a complete loss of the Hsp21's chaperone activity *in vitro* (Harndahl et al., 2001). The sulfoxidized Met residues are then reduced by a specific methione sulfoxide reductase (Msr), which restores the protein's chaperone activity (Gustavsson et al., 2002). Also, substitution of the conserved Met residues in the Hsp21 protein by oxidation-resistant Leu residues maintained chaperone functionality *in vitro*, but it is unknown if the Leu-substituted protein would function *in vivo* (Gustavsson et al., 2001).

The conservation of the Met residues suggests a specific purpose, but the data are unclear. When Met is oxidized to methionine sulfoxide (MetSo), most proteins change conformation or activity (Hawkins and Davies, 2005). At this stage, another group of proteins called Msr proteins play the role of reducing the oxidized Met (Rouhier et al., 2006). Two types of Msr proteins have been identified and named MsrA and MsrB proteins. In *Arabidopsis*, there are 14 total Msr proteins (5 MsrA and 9 MsrB) that are found in the cytosol, chloroplasts and secretory pathway, as shown in Table 2.

Gene	Accession #	Localization
AtMSRA1	At5g61640	Cytosol
AtMSRA2	At5g07460	Cytosol
AtMSRA3	At5g07470	Cytosol
AtMSRA4	At4g25130	Chloroplast
AtMSRA5	At2g18030	Secretory Pathway
AtMSRB1	At1g53670	Chloroplast
AtMSRB2	At4g21860	Chloroplast
AtMSRB3	At4g04800	Secretory Pathway
AtMSRB4	At4g04810	Cytosol
AtMSRB5	At4g04830	Cytosol
AtMSRB6	At4g04840	Cytosol
AtMSRB7	At4g21830	Cytosol
AtMSRB8	At4g21840	Cytosol
AtMSRB9	At4g21850	Cytosol

Table 2: List of Msr Proteins in *Arabidopsis.*

The proteins have been determined to be more highly expressed when plants encounter conditions that produce oxidative stress (Vieira Dos Santos et al., 2005). The production of these proteins at various growth stages or during stress is potentially very important to the viability of the organism. An example of such activity is the lack of Msr expression in Alzheimer's disease patients (Gabbita et al., 1999). Msr activity has also been linked to factors such as life expectancy, but the true extent of Msr importance is unknown (Moskovitz et al., 1995). In *Arabidopsis*, various Msr proteins have been studied for their expression during water stress, photo-oxidative treatment, and biotic stresses. Surprisingly, only oxidative damage produced an increased expression of Msr proteins (Vieira Dos Santos et al., 2005). Due to chloroplast specificity, MsrB1 and

MsrB2 proteins were chosen to study their possible interaction with Hsp21 during oxidative stress. Since MsrB1 and MsrB2 levels were are localized to the chloroplasts, they could play a role in fighting reactive oxygen species produced by photosynthesis.

Hsp21 and Msr proteins share similar roles in the protection of organisms from many different stresses. In *Arabidopsis*, we will undertake a study of how Msr proteins may interact or play a role in plant protection along with Hsp21. Homozygous, backcrossed lines of Hsp21 mutant line 10-4 at the splice site will be analyzed for a heat induced phenotype and once understood, MsrB1 and MsrB2 knockout plants will be used to determine physiology of the cell for viability amongst oxidative and heat stresses.

At the moment, the splice site mutant has been backcrossed to wildtype as the first step in securing homozygous mutants free from background mutations, given that the TILLING mutants are highly mutagenized. Recent purification of HSP21 will also allow for crystallization and structure identification. Also, a double knockout MsrB1/ MsrB2 mutant, as well as each single mutant has been assayed for heat tolerance hypocotyl elongation and 10 day light treatments. Preliminary data suggest that the MsrB1/B2 double knockout and MsrB1 have decreased growth during heat stress (see Figure 7).

Materials and Method

Genetic Material and Wildtype *Arabidopsis*

Standard *Arabidopsis thaliana* ecotype Columbia-0 was used in comparison to mutant lines. Mutant lines for the splice site mutant (10-4) were obtained by TILLING mutagenesis. The TILLING (targeting induced local lesions in genomes) mutants are used successfully for high-throughput screening of libraries mutagenized with EMS.

From this method, we obtained 18 point mutations within the Hsp21 gene. Of these, 6 were chosen specifically based on their sequence position. The splice site mutation was the only one consistently shown to be a null mutant for Hsp21 (Jennifer Corrigan).

Seed Sterilization and Plant Growth

To sterilize seeds, *Arabidopsis* seeds were hydrated in 0.6mL water for 30 min on a rotator, followed by sterilization by incubation for 5 min with equal volumes of bleach. Sterilized seeds were planted by pipette on 10mL of PNS media in 10 cm square petri dishes. To stimulate germination, plates were wrapped with parafilm and placed at 4°C for 3 days. Seeds were then grown in the dark at room temperature for 2.5 days before moving to a light incubator for 16hr light per day. Approximately 1.5 weeks later seedlings were transferred to soil and grown under same 16hr day conditions.

Hypocotyl Elongation Assay

After standard planting procedures, plates were placed vertically in the dark at 22°C. After 2.5 days, growth was marked and the plates subjected to various heat stress conditions: 22°C, 38°C pretreatment (90min), 38°C (pretreatment) plus 2hrs 22°C followed by 45°C for various times, or 45°C for 2 hrs. Height of seedlings was marked and plates were returned to the dark and grown vertically for another 2.5 days at which growth was marked again and measurements of growth taken both before and after stress.

7-10 Day Light Assay

After 3 days incubation at 4°C, seeds on plates were placed directly into standard light conditions of 16hr light/8hr dark. After 10 days of growth, stress was administered as for the hypocotyl elongation assay. Plates were returned to growth conditions for a further 6 days at which time they were scored for survival.

DNA Isolation and Genetic Screening

Genomic DNA was extracted from leaves of seedling plants using standard methods. In the case of tilling point mutations, the gene of interest was amplified by PCR and sequenced to determine genotype. The resulting DNA was observed after agarose gel eletrophoresis separation to determine proper amplification. Resulting sequenced PCR product was approximately 800 base pairs long as designed, using primers Hsp21_F and Hsp21_R.

HSP21 Purification for Crystallization

Available vectors designed to express the mature, 21 kDa form of Hsp21 (plasmid AZ#376) were transformed into *E. coli* Rosetta2 cells treated for competency. The cells were grown in 1L 2XYT with 100μg/ml ampicillin until the culture reached an optical density of ~0.4-0.8 at 590 nm. The cells were then induced by addition of 1 mM IPTG overnight. Cells were pelleted by centrifugation (14000 rpm for 5 min) and resuspended in 25mM Tris, 1 mM EDTA, pH7.5, 1 mM benzamidine, 5 mM ε-amino-n-caproic acid buffer. Cells were lysed by sonication with 5x30 second pulses while on ice. Benzonase nuclease (25U/ml) and $MgCl_2$ (2mM final) were added and the sample was centrifuged again. $(NH_4)_2SO_4$ precipitation was carried out on the supernatant with four cuts taken: 0-45%, 46-60%, 61-80%, and 81-100%. Hsp21 was identified in large quantities in both the second and third cut (from 46-80%), but was more pure in the third, or 61-80%. Both cuts were used to acquire a large concentration of protein. Each cut was individually separated through both a 25ml bed volume Macroprep DEAE-Sepharose column and a Sephacryl-300 column on an AKTA Prime FPLC. Fractions were taken across the peaks and after both columns. Hsp21 containing fractions with minimal contamination were collected,

pooled and concentrated. The final concentration was determined by using the extinction coefficient at 280 nm. The final volume was approximately 6ml with a concentration of 5.78mg/ml, giving a final field of 34.68 mg of Hsp21 in 2L of cells.

Results:

Location and Status of the TILLING Mutations

The Hsp21 protein is the only sHsp located in the chloroplast and is of particular interest to determine its importance. We obtained a set of tilling mutations (point mutations) located at different sites within the Hsp21 gene. Six mutants were of particular

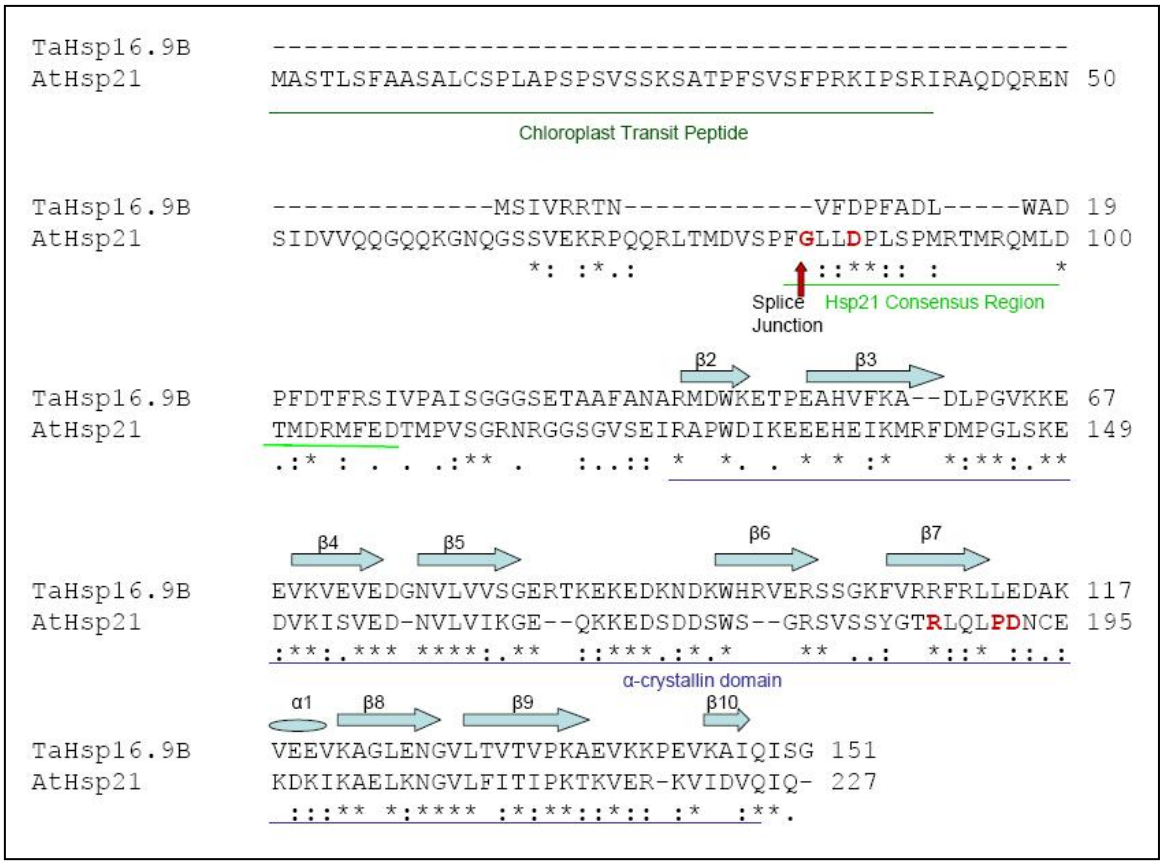

Figure 4: Position of the tilling mutations on a sequence alignment of AtHsp21 to TaHsp16.9B.

Alignment of AtHsp21 with TaHsp16.9B showing structural features and conserved residues. (*-Identical, :-conserved substitution, .-semi-conserved substitution). Positions of the mutations in the six tilling lines are shown in red. (Figure from Jennifer Corrigan)

interest because of their possible phenotypic significance due to their locations. Figure 4 above shows the positions of the six mutations in red on the coding sequence of Hsp21. Five mutations resulted in an altered amino acid, while the sixth mutation was conversion of the 3' splice site of the Hsp21 intron from a guanine to a cytosine. The arrow in Figure 4 shows the position of the intron. These mutations were identified and categorized by Jennifer Corrigan and used for initial screening.

sHSP21 Mutation	Status
Splice	Backcrossed, Awaiting Confirmation as of 5/3/08
G84E	1st Backcross Completed, Currently Awaiting Confirmation
D87N	Awaiting 1st Backcross
R187Q	1st Backcross Completed, Currently Being Selfed for Homozygotes
P191S	1st Backcross Completed, Currently Being Selfed for Homozygotes
D192N	Awaiting 1st Backcross

Table 3: Current status of all original TILLING mutations.

The 6 mutants were previously backcrossed but later analysis shows many crosses were unsuccessful. Table 3 shows their current progress. The splice site mutation was focused on directly and determined to be especially difficult to backcross because of poor germination and growth of homozygous mutant plants. The large amount of background mutations in TILLING mutants has made backcrossing difficult but a backcross was recently performed and must be confirmed by sequencing.

Hsp21 Expression in the Splice Junction Mutation

The mutation at the splice junction (named 10-4) was the focus of this study because it is predicted from previous studies to be null for expression of Hsp21. After repeated western analysis by isolating heat induced (38° for 90 minutes and isolated after an additional 120 minutes recovery time) and non-heat stressed plant protein, we have

concluded that homozygous 10-4 mutation in the splice site has indeed an effect on Hsp21 expression, producing either very little, or a complete lack of protein. The western blot of this expression is shown in Figure 5, as well as the corresponding genetic sequence for the plants being tested, indicating that the heterozygous and homozygous mutations of 10-4 have different expression levels of Hsp21. Clearly, there is very little HSP21 in HS 10-4 plants.

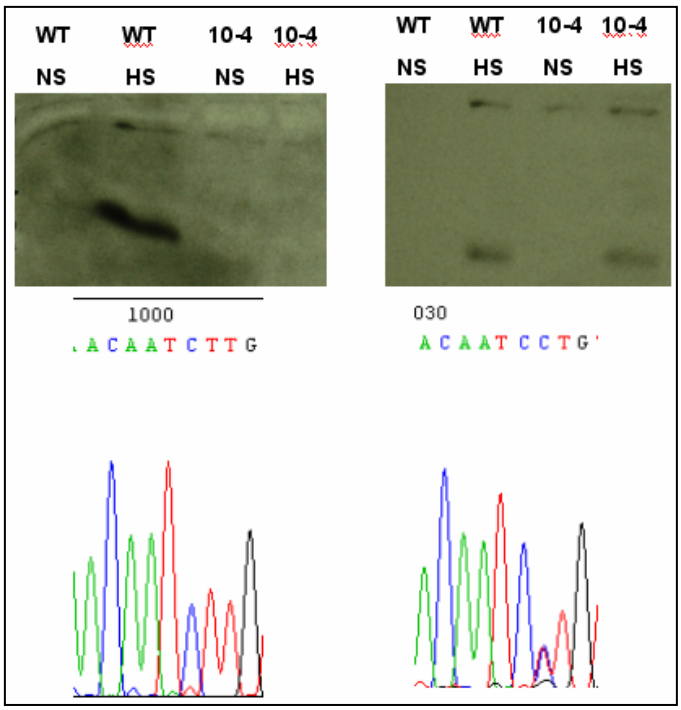

Figure 5: Western Blot Analysis of Heat Stress Plant Protein

Arabidopsis plant protein was isolated after heat stress and western blotted for quantify Hsp21 expression in the Tilling mutant 10-4. Here we focus on the splice site and the control, with it being clear that the splice does, and is confirmed to lack Hsp21 expression when in the homozygote mutant form. The left panel shows a homozygous mutant, with no Hsp21 expression, which the right shows a heterozygous mutant with some expression of Hsp21.

<u>Thermotolerance and Hsp21 Expression in MsrB1 and 2 Mutants.</u>

Additional preliminary data has been obtained for the *msrB1/B2* double knockout and *msrB1* and *msrB2* single knockouts in terms of a hypocotyl elongation assay. Graph 1 shows that unbackcrossed plants may show a phenotype in that B1/B2 double knockouts are smaller and show decreased growth after stress. *msrB1* also seems to share this phenotype. These plants were measured by means of three variable stresses including a control at 22°, 38° for 90 minutes, and lastly 38° for 90 minutes, recovery at 22° for 90 minutes, and 45° for 180 minutes. The last condition was enough to almost entirely kill *hot1-3* plants, as is their known phenotype. Under this condition it is possible to notice the phenotypes exhibited

by the *msrB1/B2* double knockout and possibly the *msrB1* single knockout as well. This assay has been repeated but results were not reproducible. However, a backcrossed version of these mutants may provide more reproducibility. In Figure 6 below, the gene structures with coding regions are T-DNA mutations (in *MsrB1/MsrB2* are shown with arrows).

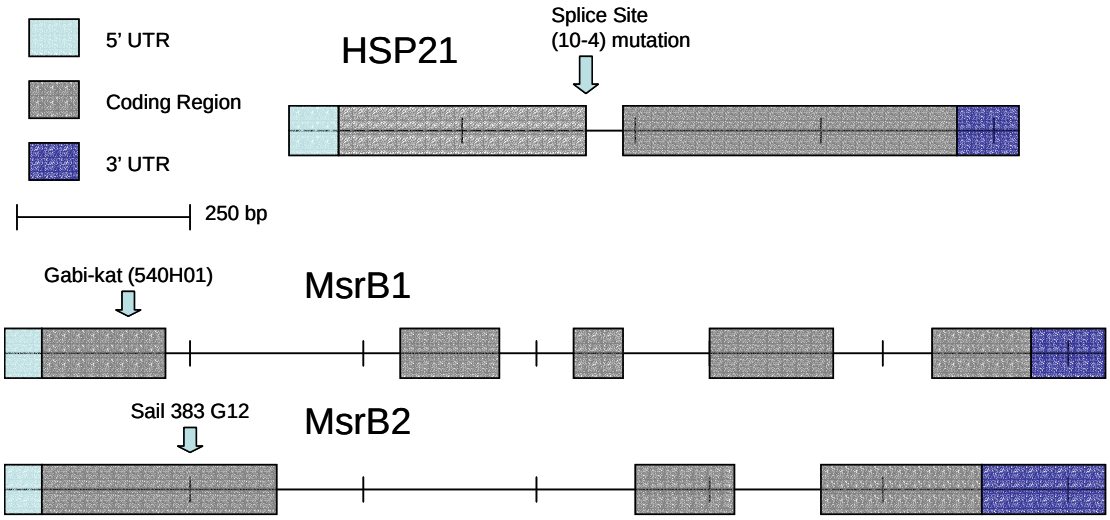

Figure 6: Gene Structure of HSP21, MsrB1, and MsrB2, showing coding regions, and indicating sites of mutation or insertion.

In addition, after 10 days of growth in constant light, the *msrB1/B2* double knockout and possibly the *msrB1* single knockout were expected to show some phenotype after heat stress. Again, these results were mixed and a fully backcrossed version may solidify conclusions. Regardless, there was initial no indication of a phenotype in the light assay and but the results for the dark assay can be viewed in Figure 7 below. Results are inconclusive about whether or not Msr mutants have varying levels of Hsp21, as several westerns showed different phenotypes. The next step would include a sequencing step along with western analysis to analyze homozygous mutants.

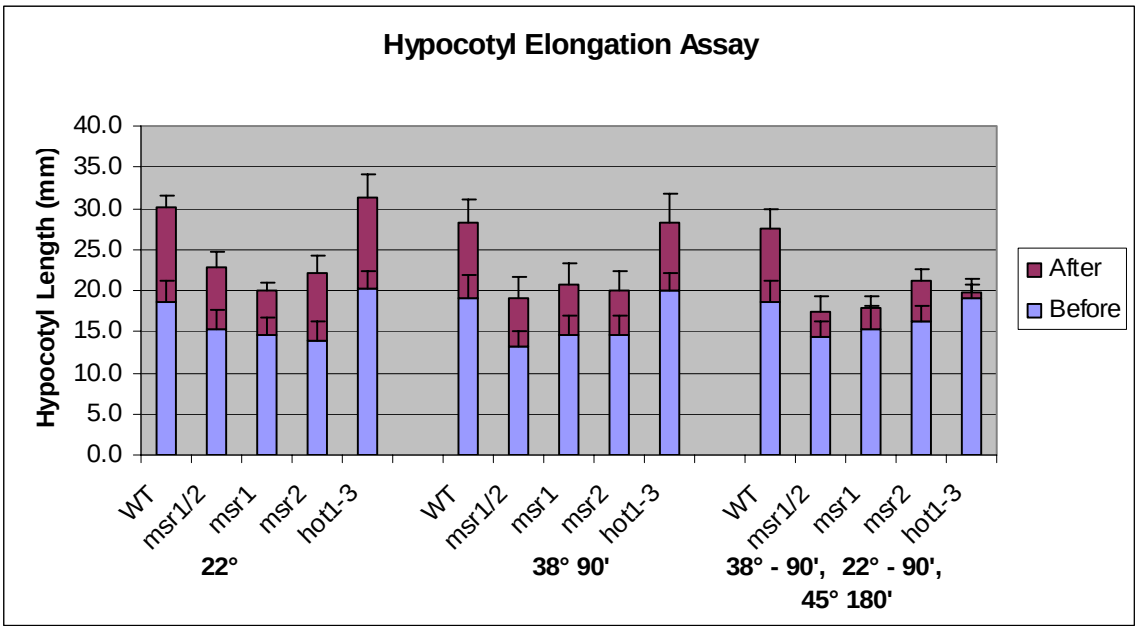

Figure 7: Hypocotyl Elongation Assay for Msr Proteins

MsrB1/B2 double knockout and individual knockouts were tests for growth after heat stress at varying time points. The growth is indicated as either before stress, or after stress, and shows the standard deviation with error bars.

Purification of Hsp21

The final volume of purified *Arabidopsis* Hsp21 was approximately 6ml with a concentration of 5.78mg/ml, giving a final field of 34.68 mg of Hsp21 in 2L of cells. The final protein was shown by gel electrophoresis to have very little other proteins but slight degradation (not shown). Protein was aliquoted to 100 ul tubes and stored for long term use in the -80°.

Discussion:

The expression and regulation of Hsps is critical to the successful viability of most organisms. Consequently, these proteins play a variety of roles in the repair and recovery of cells during stress, in particular, heat stress. With such an important role, it is not surprising that their function has been conserved across organisms from bacteria to

humans. However, much is yet to be discovered about the specific functions of individual Hsps. In *Arabidopsis thaliana* the characterization of all 19 sHsps is fundamental to the understanding of the heat stress response and to defining which proteins protect from which stresses. In this sense, we would be able to elucidate the importance that Hsp21 may have due to its localization.

Although complex, the compartmentalization of sHsps in Arabidopsis allows us the ability to study them individually relative to their location. This idea is of particular importance in the choosing to study HSP21, as it is the only sHsp localized to the chloroplast. Due to photosynthesis and other energy-related activities which occur in the chloroplast, we would expect the lack of Hsp21 to dramatically affect the stress recovery in plants. Unlike the cytosol, which contains 11 sHsps, Hsp21 may carry a unique function that may not be compensated for by other sHsps. In order to test Hsp21 function we sought to identify a a mutant lacking this protein. Results presented here indicate that the tilling line 10-4, with a slice junction mutation, fails to synthesize significant levels of Hsp21. Therefore, this mutant should provide excellent material to study Hsp21 function. However, before beginning extensive phenotypic testing, it is essential to complete at least 3 successful backcrosses to eliminate background mutations present in the initial line. A mutant with true homozygous nature is also of utmost importance because homozygous mutants show decreased expression of HSP21. Difficulty in identifying homozygous plants and obtaining significant homozygous seed have prevented further analysis of phenotypes in assays such as 10 day light or hypocotyl elongation assays of heat tolerance.

While the backcrosses were in progress, in order to conduct further assays, Arabidopsis Hsp21 was purified in its native state, to be used for crystallization trials with previously tested conditions. We hope to use the crystals to determine a structure of Hsp21 in order to comparethe secondary, tertiary, and quaternary structures to other sHsps and in other proteins of known function. Folding characteristics can reveal much about the subunits and how they may interact with other proteins or how the protein interacts with other related subunits within the protein itself. Along these lines, the orientation of the Met bristles is also of interest

The presence of conserved Met residues in a predicted amphipathic alpha-helix has led us to investigate methionine sulfoxide reductase proteins (Msr) that normally play a role in oxidative stress. Preliminary western analysis suggest that MsrB1/MsrB2 knockout plants accumulate less HSP21 under heat stress, but results indicate inconsistencies. This conclusion has not been confirmed by both demonstrating the presence of the T-DNA insertion and having backcrossed mutants for protein on the same plants. The effect of the B1/B2 knockout on HSP21 during heat and oxidative stress will be explored also when the 10-4 mutation is available as a backcrossed homozygote. It has been proposed that because B1 and B2 are localized in the chloroplast, and they play a role in protecting chloroplasts from radical oxygen species by reducing oxidized methionines. Lack of B1 and B2 under heat stress may lead to irreversible oxidation of Hsp21 methionines, leading to a non-functional protein. Viability of the MsrB1/B2 knockout plants will be explored under stress conditions. Initially, MsrB1/B2 and MsrB1 have been shown to have decreased growth via hypocotyl elongation assays, but results are inconclusive. Also, inconclusive results have been obtained as to their response to

heat stress as 10 day old light grown plants. I will also be most important to compare these results to a plant lacking HSP21 in the chloroplast.

The combined study of the MsrB proteins and Hsp21 Splice junction (10-4) mutation will eventually help discover the pathways that may be involved in increased tolerance to various stresses. *In vivo*, sHsps are highly complex and each specific protein function that is understood will further elucidate how these crucial proteins provide organisms with such incredible sustainability.

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