Genetic Analysis of an Enzyme Involved in Nitric Oxide Metabolism in *Arabidopsis thaliana.*

By

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ABSTRACT

S-nitrosoglutathione reductase (GSNOR) is an enzyme, encoded by the *ADH2*/*HOT5* gene in *Arabidopsis thaliana*, which is involved in nitric oxide metabolism in the cell. Nitric oxide is not only important in plant growth and development, but is also a key signaling molecule in all organisms. GSNOR is a highly conserved type III alcohol dehydrogenase that is responsible for breaking down S-nitrosoglutathione (GSNO) into GSSG and NH_3, an NADH-dependent reaction. We have found that regulation of this reaction by GSNOR is crucial for survival of heat stress. Mutated, nonfunctional forms of GSNOR have been identified, including the *hot5-1* mutation, a missense mutation that changes glutamate 283 to lysine. Although the *hot5-1* protein accumulates, dark-grown seedlings with this mutation die after heat treatment at temperatures that wild-type plants can survive. To study further the importance of GSNOR in plants, a screen for suppressors of *hot5-1* has been initiated. Additional mutations were chemically induced in the *hot5-1* mutant background and seedlings screened to find mutations that restore the heat-tolerant phenotype to *hot5-1*. Primary screening of over 50,000 seedlings has been completed. Five promising mutants have been identified, designated 4-4c, 6-3a, 20-1b, 23-1b and 35-2c. The *HOT5* genomic region of these five mutants has been sequenced confirming that all still contain the original *hot5-1* mutation. Furthermore, the remainder of the gene is wild type, indicating that the suppressor mutation(s) responsible for the thermotolerant phenotype are extragenic. Future work will involve map-based cloning to identify the suppressor loci. Identity of suppressor genes should provide new insight into the regulation of nitric oxide and nitrosative stress in plants.

INTRODUCTION

The free radical nitric oxide (NO) has been shown to be a very important membrane diffusible signaling molecule in higher organisms (1). In plants, it is responsible for promoting seed germination, plant greening, flowering, defense responses, stomatal closing and can also inhibit hypocotyl elongation (1-3). As a signaling molecule, NO has been shown to be important in protein nitrosylation, nitrosation or nitration for various regulatory purposes.

The pathway(s) of NO production in plants has not been completely resolved. Nitrate reductase (NR) has been established as an enzyme that can catalyze the NADPH-dependent conversion of nitrite to NO, though its primary recognized function is to convert nitrate to nitrite (1,4). However, it has yet to be determined if the NO produced from NR is biologically active, and if the plant can distinguish between different sources of NO for use during signaling (3). NR is an inducible protein, depending on the availability of nitrate and light; it has been shown that activation and regulation of NR is closely related to photosynthesis, presence of sugars, cytosolic acidification and anaerobiosis (3). Additionally, it seems that NR production follows a circadian clock: high levels of NR mRNA transcript have been observed late at night, before early morning (3).

The regulation of NR activity has been studied in some detail; divalent cations, protein kinases, protein phosphatases and 14-3-3 binding proteins have all been shown to have post-translational regulatory action on NR (4). Specifically, it has been shown that two important steps inactivate NR: phosphorylation on a specific serine residue and subsequent interaction with a 14-3-3 protein (3,4). The rate of degradation is much higher for the inactive form of NR compared to the active form, and degradation also occurs more quickly in the dark (4).

Another enzyme that has been suggested for NO formation in plants is nitric oxide synthase (NOS). However, it has not been proven that plants utilize a NOS enzyme for the production of NO. In mammals, three different forms of NOS (inducible, endothelial, and neuronal NOS) are all responsible for oxidizing L-arginine to N-hydroxyarginine and then to NO and citrulline (1). Endothelial and neuronal NOS enzymes are expressed constitutively, and are grouped under one common name, cNOS. These NOS enzymes are activated by calmodulin and increases in calcium concentration (1). Inducible NOS (iNOS), however, is always bound to calmodulin and is not regulated post-translationally. Animal NOS proteins range from 130 kDa to 160 kDa in size, form dimers, and are approximately 50-60% identical in mammals (1).

While no protein in plants has yet been identified as a NOS-like enzyme, another enzyme, christened AtNOA1 (for nitric oxide associated 1), has been shown to be involved in NO synthesis or accumulation *in vivo* (5). At this time, the exact function of AtNOA1 has yet to be elucidated but the enzyme has been shown to be needed for normal growth and development in *Arabidopsis*. The gene product for AtNOA1 was purified and analyzed, and found to have kinetic values of $K_m = 12.5$ μM for arginine and $V_{max} = 5.0$ nmole min^{-1} mg^{-1} (6). The purified enzyme, in addition, was shown to have activity dependent on $NADP^+$, CaM, and Ca^{2+} and inhibited by N^G-nitro-L-Arg-methyl ester (L-NAME), which is known to inhibit eNOS and nNOS in mammals (6). However, there are some conditions in which AtNOA1 behaves nothing like any known NOS enzyme, so at this time it is being classified only as a nitric oxide associated enzyme.

A protein referred to as S-nitrosoglutathione reductase (GSNOR) has been identified as another important player in NO metabolism in *Arabidopsis*. GSNOR is encoded by the *ADH2*/*HOT5* gene in *Arabidopsis thaliana*, and is a highly conserved class III alcohol

dehydrogenase (ADH). GSNOR is responsible for breaking down S-nitrosoglutathione (GSNO) to oxidized glutathione (GSSG) and ammonia, an NAD/NADPH-dependent reaction. When kinetically analyzed from rat liver cytosol, this reaction was found to have a K_m of 0.028 mM at pH 7.4 and a k_{cat} equal to 2,640 min^{-1} (7). These values give a catalytic efficiency of k_{cat}/K_m equal to 94,300 $mM^{-1}•min^{-1}$, a number considerably larger than that of other ADH class III enzymes analyzed (7). This indicates that GSNO is one of the most active substrates for GSNOR, a class III ADH (7). Characterization of GSNOR activity was also completed in 25-day-old wild-type Arabidopsis plants and was found to be 12.1 ± 1.3 $nM•min^{-1}•mg^{-1}$ of total cell protein (8).

It has been shown that NO is an important signaling molecule required for normal growth and development in plants (9), but we have additionally found that SNO regulation by GSNOR is crucial for survival of heat stress (8). We have isolated four mutant alleles of the *HOT5* gene, two T-DNA insertion null alleles *hot5-2* and *hot5-4*, and two missense alleles *hot5-1* and *hot5-3* (8). The *hot5-1* missense mutation, which is the subject of this study, results in the change of amino acid 283 from a glutamate to lysine (Figure 1, 2). The *hot5-1* mutant protein accumulates at levels approximately 30% of wild type and GSNOR activity in *hot5-1*mutants is correspondingly 33.8% of wild-type activity (8). Dark-grown seedlings with the *hot5-1* mutation die after heat treatment at temperatures that wild-type seedlings can survive. However, this heat-sensitive phenotype is only observed when *hot5-1* seedlings are grown and stressed completely in the dark; wild-type thermotolerance is regained when mutant seedlings are grown in the light. Additionally, dark-grown *hot5-1* seedlings have been imaged for nitroso species using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) stain and have been found to accumulate NO in higher levels than wild-type plants (Figure 4, (8)). The heat-sensitive

phenotype can be rescued by NO scavengers and enhanced in wild-type plants with NO donors, indicating that higher NO levels contribute to the heat sensitivity of the *hot5-1* mutant (8).

Of the other three mutants, *hot5-2* and *hot5-4* are T-DNA knockout mutants while the *hot5-3* allele is a missense mutation that results in a G288R substitution. In hypocotyl elongation assays, *hot5-3* showed similar thermotolerance to *hot5-1* mutants. The *hot5-2* and *hot5-4* mutants can only be grown on soil in the light since their hypocotyls do not elongate on minimal media plants (PNS plates) in the dark; thus, to test the thermotolerance of *hot5-2* and *hot5-4*, a leaf-disk assay was used (see Reference (8)). In this leaf-disk assay, *hot5-2* and *hot5-4* were dramatically less thermotolerant than either *hot5-3*or *hot5-1*, and showed considerable leaf yellowing. The conclusion from this assay was that *hot5-1* and *hot5-3* are relatively weak alleles compared to the T-DNA knockout mutants (8).

In order to study further the importance of GNSOR function in plants, a screen for suppressors of *hot5-1* has been initiated. Additional mutations where chemically induced in the *hot5-1* mutant background and seedlings screened to find mutations that restore the heat-tolerant phenotype to *hot5-1*. Identity of any suppressor genes isolated from this screen should provide new insight into the regulation of NO and nitrosative stress in plants.

MATERIALS AND METHODS

Mutagenesis of *hot5-1*

Chemical mutagenesis via ethyl methanesulfonate (EMS) was performed by Dr. Ung Lee on approximately 5,000 homozygous *hot5-1* seeds, representing the M1 generation. These plants were grown to maturity and M2 seeds collected in a single pool and used for the experiments reported here.

Seed Planting

Seeds were first hydrated for 20 min on a circular rotator in 0.7 mL of sterile water. Once hydrated, 0.7 mL of bleach was added for 3 min for surface sterilization. Following incubation, seeds were washed 5 times with sterile water and 5 rows of 12 seeds per row were planted on 10 cm square petri plates poured with 10 mL of PNS media (15mM sucrose, 5mM KNO_3, 2mM $MgSO_4$, 2mM $Ca(NO_3)_2$, 50mM FeEDTA, 2.5mM KPO_4, 70µM H_3BO_4, 14µM $MnCl_2$, 0.5µM $CuSO_4$, 1µM $ZnSO_4$, 2µM Na_2MoO_4, 10µM NaCl, 1µM $CoCl_2$, and 7.5g/L plant agar).

Once planted, plates were wrapped in tinfoil to keep seeds in the dark. Plates were stored at 4^{o}C for 3 days, moved to 22^{o}C for germination and 2.5 days of vertical growth in the dark before use in the hypocotyl elongation assay.

Hypocotyl Elongation Assay (HEA)

After 2.5 days growth in the dark at 22^{o}C, seedlings were exposed to heat stress conditions as follows: 1.5 hours at 38^{o}C, 2 hours at 22^{o}C, 2 to 2.5 hours at 45^{o}C. Plates were unwrapped briefly to mark hypocotyl growth directly after the 45^{o}C treatment. Plates were then rewrapped and placed vertically at 22^{o}C to grow an additional 2.5 days. At this time, plates were unwrapped and hypocotyl growth again marked. Scaled pictures were taken of the plates and hypocotyl growth measured using the program ImageJ.

DNA isolation and Genotypic Analysis

Genomic DNA was isolated from seedling plants by grinding a small leaf, approximately 1 cm square, with 200µL of DNA extraction buffer (250mM NaCl, 25mM EDTA, 2.5% SDS, 200mM Tris/HCl pH 7.5). Tubes were centrifuged for 2 min and 200µL of isopropanol added.

The tubes were vortexed and allowed to sit at room temperature for 20 min. The tubes were centrifuged then for 6 min at 4°C at 13,000 rpm. The liquid was removed from the tubes and 500µL of cold 70% ethanol added. The tubes were centrifuged again for 6 min at 4°C at 13,000 rpm. The ethanol was removed and tubes were left open at room temperature for 2 hrs to allow complete evaporation of ethanol. The DNA was resuspended in 50µL of sterile water.

DNA samples were amplified by PCR for confirmation of DNA isolation and also for submission for sequencing of the *HOT5* gene region (see Table 1 for primer sequences and Table 2 for PCR program). Two primer sets were used to amplify the entire *HOT5* region and the resultant samples were purified using a Qiagen QIAquick PCR purification kit prior to DNA sequencing. DNA sequencing was performed by the University of Arizona Biotechnology facility.

SDS-PAGE and Protein Blot Analysis

Protein samples were prepared by grinding 0.4g of seedling plant material with 0.4mL of 2X sample buffer (120mM Tris pH 8.0, 120mM DTT, 4% SDS, 30% sucrose, 10mM ε-aminocaproic acid, 2mM benzamidine, 0.02% bromophenol blue). Once plant material was homogenized, samples were boiled for 5 minutes and centrifuged to remove insoluble plant material. Samples were quantified by spotting 2µL of each sample and six BSA standards ranging from 0.25 to 4 mg/mL onto Whatman paper. The paper was stained using Coomassie Blue then destained completely and allowed to dry. Once dry, the sample and standard spots were punched out into Eppendorf tubes containing 1mL of 2% SDS. Additionally, a blank tube was prepared using the Whatman paper background. The tubes were then allowed to sit overnight to elute the Coomassie Blue. Absorbance of the solution was measured at 595nm using

a Varian Cary 50 UV-Visible photospectrometer after blanking with the Whatman paper alone sample. Protein concentrations were calculated via the equation generated from the absorbance values of the standard samples.

Once quantified, 10µg of protein was separated by SDS-PAGE (5% stacking gel, 10% resolving gel). Gels were either stained with Coomassie Blue to ascertain the amount of protein present or were prepared for Western blotting on nitrocellulose using standard techniques. Membranes were probed using a rabbit antiserum against Arabidopsis GSNOR, and bands were visualized using an anti-rabbit horseradish peroxidase and enhanced chemiluminescence detection reagent (Amersham International) with BioMax film (Kodak).

RESULTS

Choice of Allele for the Suppressor Screen

The *hot5-1* mutant allele was chosen for this suppressor screen for a variety of reasons. It was the first mutation in the *HOT5* gene region isolated, so more advanced backcrossed material was available for this allele than for the subsequently isolated *hot5-3* allele. Secondly, the purpose of this suppressor screen is to elucidate possible proteins that are interacting with GSNOR and may contribute to thermotolerance. The null mutants, *hot5-2* and *hot5-4*, would not serve this purpose since they are knockout mutants that result in no production of GSNOR.

Initial Screen and Isolation of Putative Suppressor Mutants

In the initial screen, 53,868 M2 generation mutagenized *hot5-1* seeds were screened (963 plates with 48 or 60 M2 seeds per plate), representing approximately 5,000 M1 generation plants. Initially, *hot5-1* and wild-type seeds were planted as reference (Figure 5), but later were

eliminated in order to accommodate more M2 seeds and facilitate speed of screening. Plates of 2.5 day old dark grown seedlings were heat shocked at 38^{o}C for 1.5 hrs, 22^{o}C for 2 hrs, and exposed to 45^{o}C for 2 or 2.5 hrs. Putative suppressor mutants were identified if M2 seedlings showed growth after heat stress of at least 0.2mm more than the growth of *hot5-1* after heat stress (Figure 5). Of the M2 generation seeds screened, 898 putative suppressor mutants were isolated, giving an initial isolation frequency of 1.67% of M2 seeds screened.

Thermotolerant M2 seedlings were removed from plates and planted on soil to obtain M3 generation seeds. Of the 898 seedlings isolated, 160 died after transplantation to soil and thus could not be further analyzed ([160/898]*100 = frequency of 17.8%). Of the remaining 738 putative mutants, 383 have been harvested for M3 seeds. 51 of these did not produce any M3 seeds ([51/383]*100 = frequency of 13.3%) and again could not be further analyzed. Those M3 generation seeds that could be obtained were retested to confirm a thermotolerant phenotype using the hypocotyl elongation assay. 24 seeds from each M3 plant were tested again compared to both wild type and the original *hot5-1* mutant. Once retested, putative mutants were classified into one of three categories based on their observed thermotolerance: "yes," "maybe," or "no." Mutants classified as "yes" showed a strong thermotolerant phenotype in reconfirmation when compared to *hot5-1*for all or most of the 24 seeds tested. If classified as "yes," mutants were further analyzed as described below. "Maybe" mutants either did not show as strong phenotypes as those mutants classified as "yes" or appeared to possibly be segregating for the suppressor phenotype. "Maybe" mutants will potentially be analyzed in the future. The "no" mutants have a completely non-thermotolerant phenotype (i.e. were phenotypically identical to *hot5-1*) and were dropped from the screen. Of the 898 putative suppressor mutants isolated from the primary screen, 329 have been retested and classified: 5 as "yes," 233 as "no," 57 as "maybe," and 34

that need to be retested before final classification. The five reconfirmed mutants represent a frequency of 0.0294% ([5/16,992]*100) of original M2 seeds screened. As described above, 211 plants of the original 898 died or did not produce M3 seeds and subsequently were dropped from the suppressor screen. This leaves an additional 358 putative suppressor mutants to be reconfirmed in future experiments.

Heat Stress Phenotype of Reconfirmed Suppressor Mutants

The five mutants which were reconfirmed in the screen of M3 seedlings have been designated 4-4c, 6-3a, 20-1b, 23-1b and 35-2c. To characterize the extent of the suppression of the growth defect, the five mutants were subjected to multiple heat stress trials and quantitative data obtained on their growth both before and after heat stress compared to wild-type and *hot5-1* (Appendix 2). All five mutants show a substantially more thermotolerant phenotype than *hot5-1* (Figures 6 and 7). On average, 4-4c and 6-3a exhibit hypocotyl elongation approximately 50% of wild-type after heat stress (Appendix 1). 20-1b, 23-1b and 35-2c show approximately 70%, 80% and 60% of wild-type growth, respectively (Appendix 1). Growth phenotypes are also observed in the putative mutants before heat stress. 4-4c, 6-3a and 23-1b grow to about 50% of wild-type length. On the other hand, 20-1b and 35-2c grow as much as, or more than, wild-type before heat stress.

DNA sequence of the *HOT5*

Genomic DNA was isolated from all five suppressor mutants and the *HOT5* genes amplified by PCR with the primers in Table 1 in order to sequence the *HOT5* gene region to determine two things. Firstly, we wanted to confirm the presence of the original *hot5-1* mutation.

Secondly, sequencing of the *HOT5* region would indicate if any additional mutations, perhaps reverting effects of the original mutation and contributing to the thermotolerant phenotype, were located within the *HOT5* gene region. Sequences of the *HOT5* gene from all five of the suppressors matched identically with wild-type, with the exception of the *hot5-1* G to A mutation (Figure 8). This indicates that the mutation(s) contributing to the thermotolerant phenotype of each of the reconfirmed mutants is located extragenically.

Expression of the *HOT5* Protein in the Suppressor Mutants

The next step was to characterize GSNOR protein levels in the confirmed mutants. Plants with the *hot5-1* mutation accumulate GSNOR at approximately 30% of wild-type levels (Figure 3, (8)) and show even lower protein accumulation after heat stress. Using western blot analysis, we were able to confirm that all five of the suppressor mutants also accumulate reduced levels of GSNOR (Figure 9). This indicates that the thermotolerant phenotype of the five mutants is not due to higher levels of GSNOR protein present, but instead is due to something else. Once the suppressor mutants have been backcrossed into wild-type and *hot5-1* this experiment can be repeated to obtain more precise estimates of GSNOR protein levels.

DISCUSSION

This set of experiments sought to identify and characterize possible suppressor mutants of the thermotolerance-defective *hot5-1* mutant. After screening 53,868 M2 seeds, representing 5,000 M1 plants, five mutants have been isolated that show thermotolerant phenotypes significantly greater than the parent *hot5-1* mutant: 4-4c, 6-3a, 20-1b, 23-1b and 35-2c. Growth after heat stress for these mutants ranged from 50 to 80 percent of wild type growth after heat

stress. The *HOT5* gene region of all five mutants was sequenced and found to not contain any additional mutations besides *hot5-1*. Furthermore, the five suppressor mutants show GSNOR protein accumulation at levels similar to *hot5-1* under both control and heat stress conditions. This latter observation suggests that the suppressor phenotype is not the result of stabilization of the *hot5-1* mutant protein. In total, the suppressors isolated should provide new genetic material to better understand the regulation of GSNOR and/or the process of thermotolerance.

DAF-FM DA staining to detect levels of nitroso species was completed on 4-4c and 6-3a, and it was observed that the mutants had a reduced accumulation of nitroso species when compared to *hot5-1* (data not shown). This experiment must be repeated, but preliminarily it suggests that the recovered thermotolerance of 4-4c and 6-3a may be due to the reduced presence of nitroso species in the seedlings. Future work will involve DAF-FM DA staining of all suppressor mutants recovered.

The five suppressor mutants isolated show a number of interesting phenotypes. Only about 65% of 20-1b mutants germinate on PNS plates after 2.5 days of growth at 22^{o}C, while the other mutants show relatively normal germination rates. After 2.5 days of growth at 22^{o}C, 4-4c, 6-3a and 23-1b grow to about 50% of wild-type length. On the other hand, 20-1b and 35-2c grow as much as, or more than, wild-type in that same time frame. When grown to maturity on soil, 20-1b has a bushy phenotype and produces many bolts. Even with production of multiple bolts, however, 20-1b has an extremely low seed yield compared to both wild-type and *hot5-1*. Only one mutant, 4-4c, produces seeds anywhere near wild-type levels. Further phenotypic data will be obtained after backcrossed lines of the mutants have been established; backcrossing will be required to determine if any phenotypes cosegregate with the suppressor mutation.

The next major step for further investigation of the suppressor mutants is genetic analysis. All confirmed mutants will be backcrossed into wild-type and *hot5-1* backgrounds in order to eliminate extraneous mutations. Backcrossing will also allow determination if the mutation is dominant or recessive, and also if it segregates as a single mutation. The resultant progeny will be tested in a hypocotyl elongation assay to isolate plants that still show a thermotolerant phenotype, and characterization will again be completed. There is a small possibility that the suppressor mutants isolated are not independent mutations. Because of this, once stable backcrossed lines are obtained, the mutants will be crossed to one another in order to determine if the suppressor mutations lie within the same gene. Mutants will also be backcrossed into *hot5-2*, the null allele, and *hot1-3* (an HSP101 knockout mutant, also heat sensitive) to determine if the *hot5-1* suppressor mutations rescue other thermotolerance-defective mutants.

Future work will involve reconfirming the remaining 358 putative suppressor mutants isolated. The *HOT5* gene region of any additionally confirmed mutants will be sequenced, as described above, in order to show the *hot5-1* mutation is still present and also to determine if any additional mutations exist. GSNOR protein levels will also be investigated. DAF-FM DA staining will be completed on any new mutants isolated, as well as on the five suppressor mutants already reconfirmed. Additionally, map-based cloning will eventually be used to locate the suppressor loci. The goal of this suppressor screen is to determine more fully the GSNOR pathway of NO regulation and how it affects thermotolerance in Arabidopsis. We are expecting to eventually find proteins that either interact directly with mutated *hot5-1* GSNOR and act as suppressors, or potentially bypass the GSNOR pathway completely and result in a thermotolerant phenotype. Identity of suppressor genes should provide new insight into the regulation of NO and nitrosative stress in plants.

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TABLES AND TABLE LEGENDS

Table 1: Primer sequences used in PCR amplification and sequencing reactions. The bold and underlined letter shown in the hot5-3 R primer indicates the location of the *hot5-1* mutation.

Primer Name	Sequence (5' → 3')	Used for
New gADH-F	GAGTATATCAACTCCGCTGTC	PCR
FALDH-GABI	ACAGCCTCAAATTGATTCACTAA	Sequencing
hot5-4 F	TTCTTGGATGTGGTGTTCCCA	PCR
ghot5-2160R	TCAGTGATG	PCR
hot5-3 R	CTTGTGACAGCACT**C**CAATGC	Sequencing
New gADH-R	TCATTTGCTGGTATCGAGGACAC	PCR

Table 2: PCR reaction program.

Step	Temperature (^{o}C)	Time (minutes)
1	95	5.0
2	94	0.5
3	52	0.5
4	72	2.0
5	Repeat 40 times to Step 2	
6	72	10.0

FIGURES AND FIGURE LEGENDS

Figure 1: Location of the *hot5-1* mutation in the Arabidopsis GSNOR homodimer structure, as determined by the Montfort Lab. The green ribbon structure shows the A subunit, and the blue ribbon structure shows the B subunit. The red stick structure is NAD^+, as shown in its binding pocket. Single red spheres are zinc atoms in the active site and in a structural site. The mutation E286K is shown by the space-fill representation located near the dimer interface.

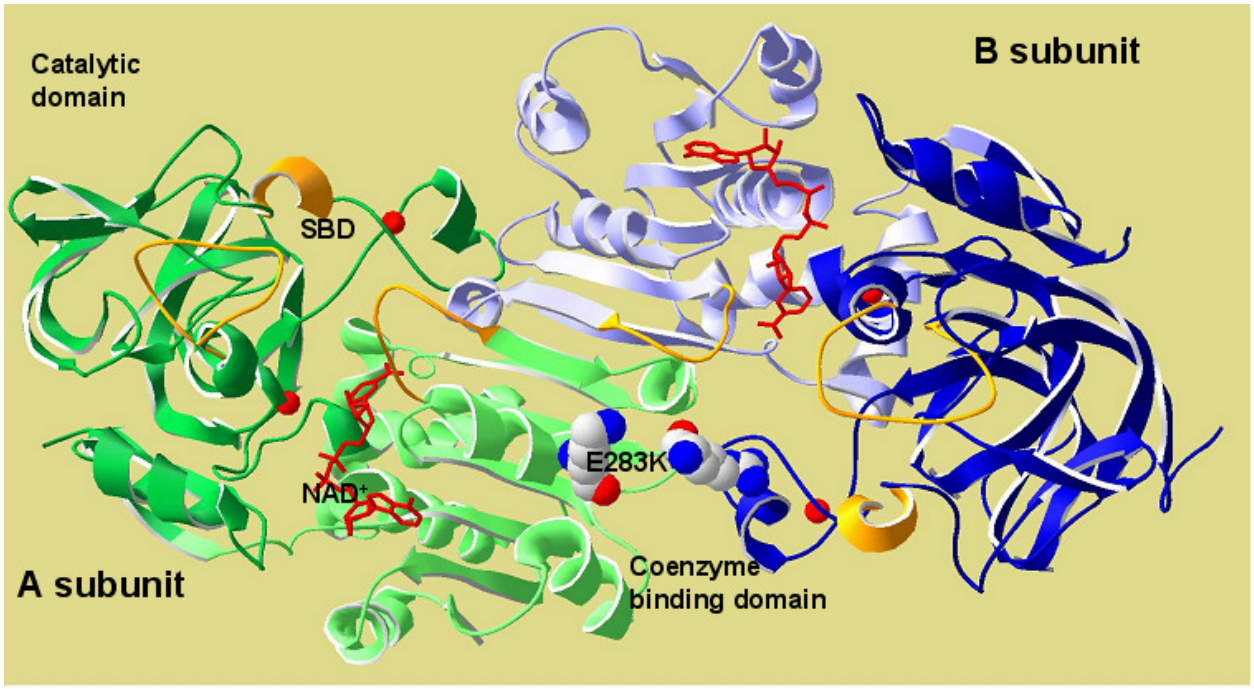


Figure 2: Amino acid sequence alignment of GSNOR from Arabidopsis (accession no. AAB06322), Rice (accession no. BAD21999), Maize (accession no. CAA71913), E. coli (accession no. NP_414890) and Human (accession no. NP_000662). Identical residues are highlighted in black and conservative replacements in gray. Secondary structure elements as seen in the *AtGSNOR* structure appear over the alignment (Dr. Montfort, unpublished data). Conserved motifs are indicated below the alignment: SBD, substrate binding domain. The two structural domains of GSNOR are colored; coenzyme binding domain, green; catalytic domain, orange. Above the sequence, α-helices are shown as solid arrow, β-strand as boxed line, and loop as straight. Positions of each of the primary *hot5* mutations are indicated above the alignment in red.

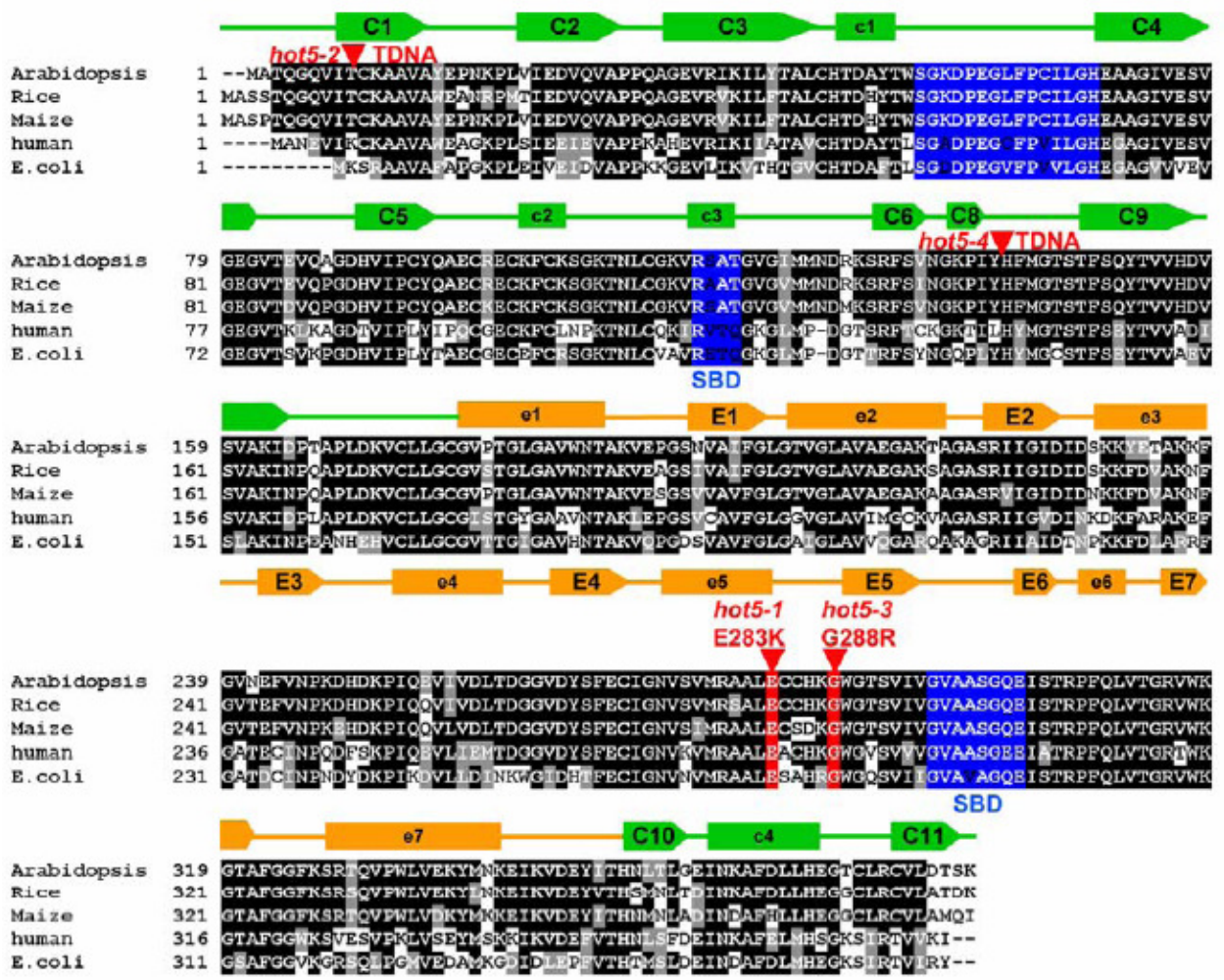


Figure 3: Accumulation of GSNOR and HSPs in wild-type and *hot5-1* mutant plants. Total protein was isolated from control (C; 22°C) or heat-stressed (H, 38°C for 90 min, followed by 2 h at 22°C) 25-day old leaf discs and analyzed with the indicated Arabidopsis HSP and GSNOR antisera. Equal quantities of total protein (0.5 mg for Hsp101 antibodies, 5 mg for GSNOR and sHSP antibodies) from each of the mutants or the wild type were separated on 7.5% (Hsp101), 10% (GSNOR), or 15% (sHSP) SDS-PAGE gels. Protein blot analysis with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies confirmed the presence of similar protein levels. Figure courtesy of Ung Lee from Reference (8).

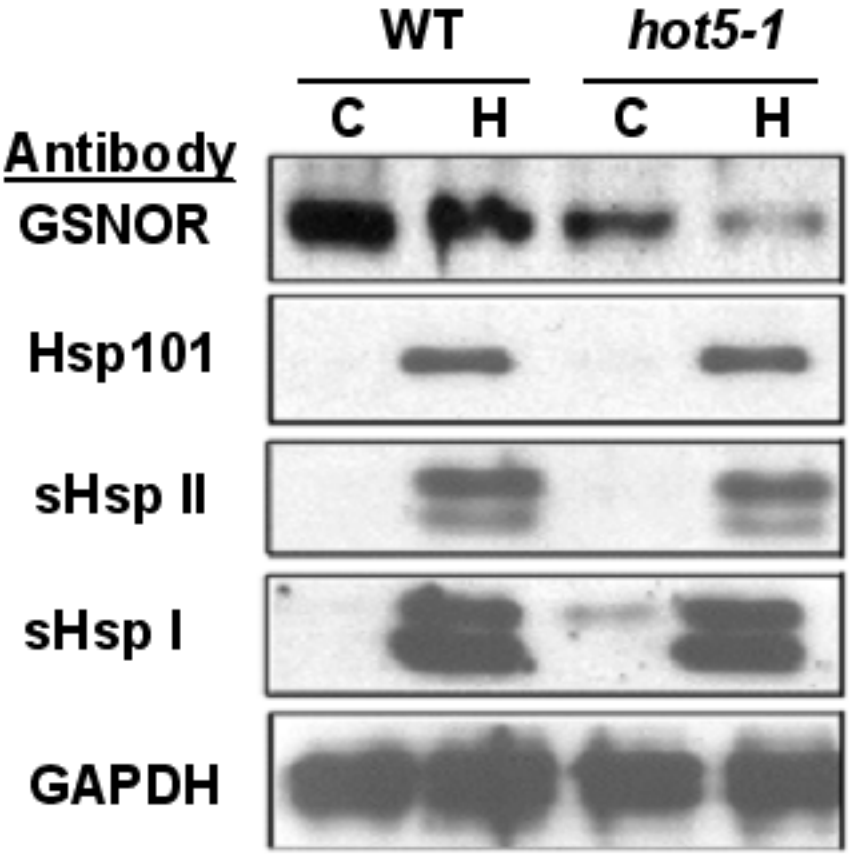


Figure 4: Nitroso levels in wild-type and *hot5-1* mutant seedlings, as imaged using DAF-FM DA stain. 2.5 day-old dark-grown seedlings were stained and visualized by confocal laser scanning microscopy using an LSM model 510 META microscope (Zeiss) equipped with a Plan-Apo 633 1.4 lens (numerical aperture). Fluorescence was captured following excitation at 488 nm and detection at 505 to 570 nm (BP505-570 infrared filter). The Zeiss LSM Image Browser 3.2 program was employed for image acquisition, and Photoshop Elements (Adobe Systems) was used for image processing. The first panel shows DAF-FM DA fluorescence while the second panel is a natural light picture indicating position of the seedlings.

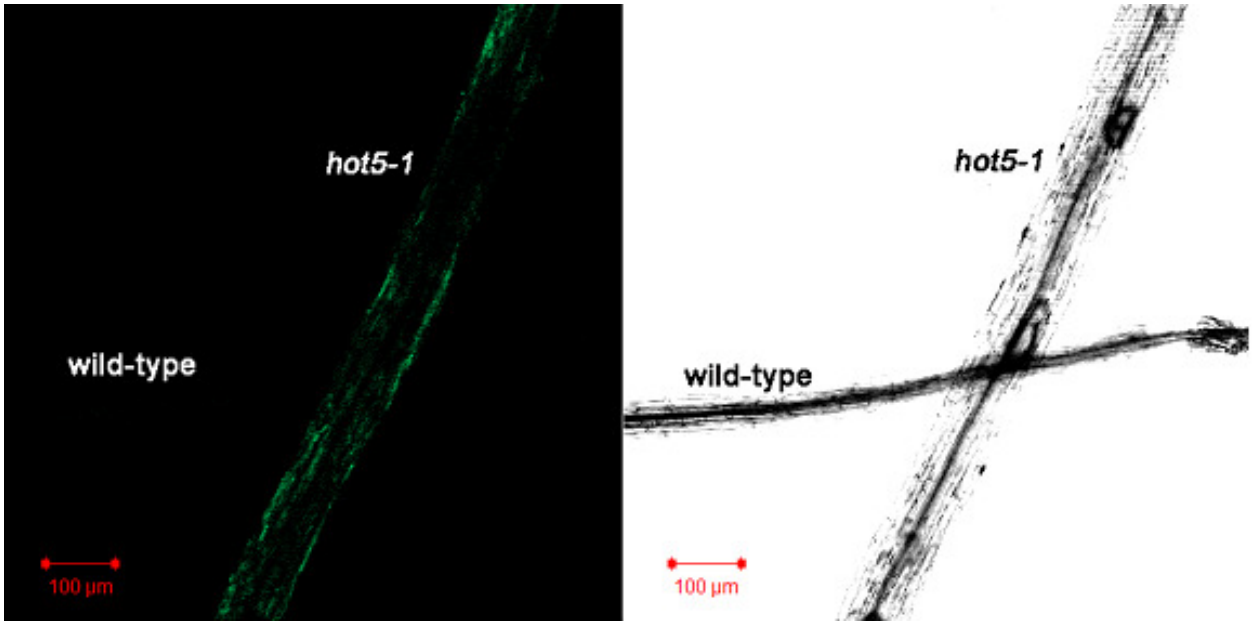


Figure 5: Isolation of putative suppressor mutants from the M2 generation of mutagenized *hot5-1* seeds. Wild-type seedlings (upper left) are labeled "col" and "*hot5-1*" (upper right) shows seedlings carrying only the original *hot5-1* mutation. The *hot5-1* M2 seeds (remainder of plate) are the seeds used for primary screening. Seedlings were marked with blue dots to indicate growth at time of heat stress. After an additional 2.5 days at 22°C, seedlings were scored for further growth with a red mark. The circle indicates a putative mutant isolated because of its thermotolerant phenotype (ability to grow after heat stress like wild-type).

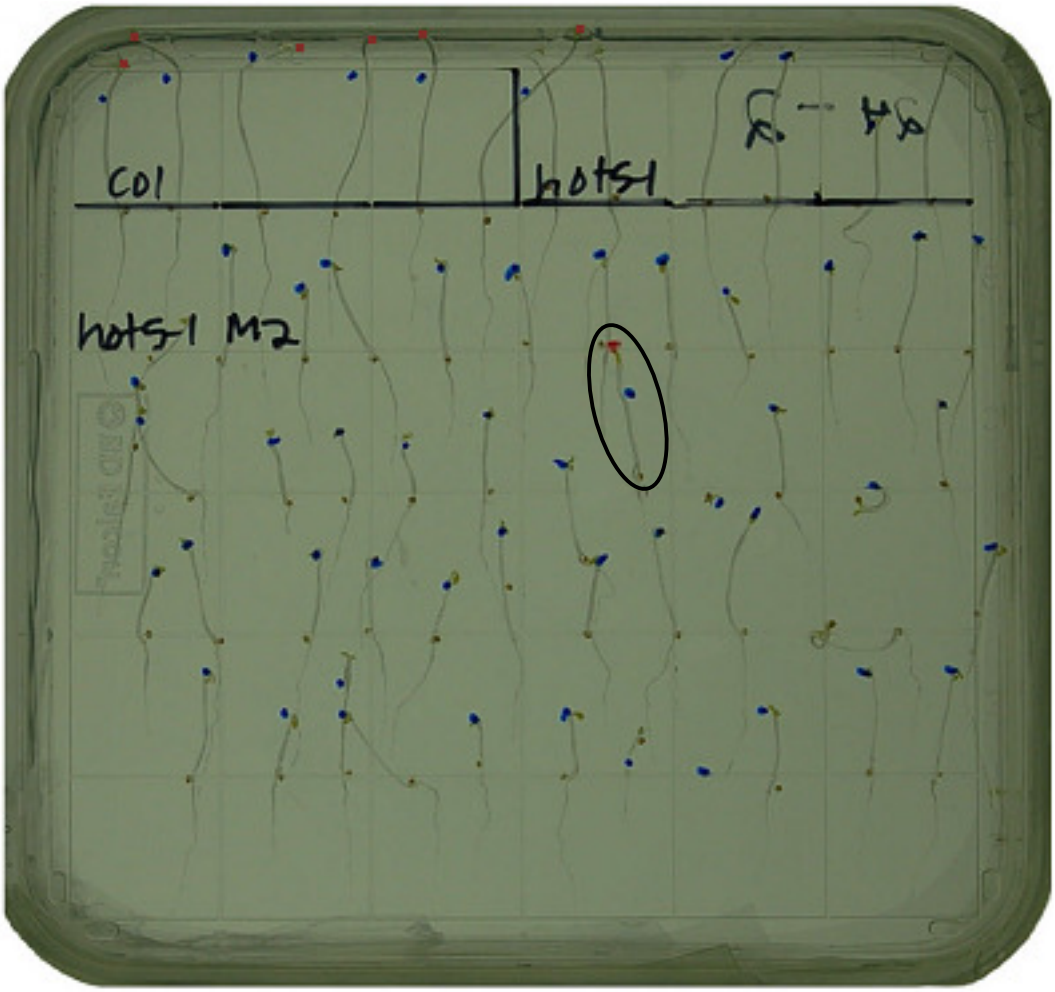


Figure 6: Average hypocotyl growth before and after heat stress for controls and two reconfirmed suppressor mutants. In each trial, 24 seeds were planted for each suppressor mutant and 6 seeds planted for both wild-type and *hot5-1*. 2.5-day-old plants were heat shocked at 38°C for 90 min, 22°C for 2 hrs, and 45°C for 2 hrs. The before heat stress (HS) data was collected directly after treatment, and after HS data was collected 2.5 days after treatment. Two trials are shown (RC2 and RC3). The error bars represent standard deviations of hypocotyl elongation measurements.

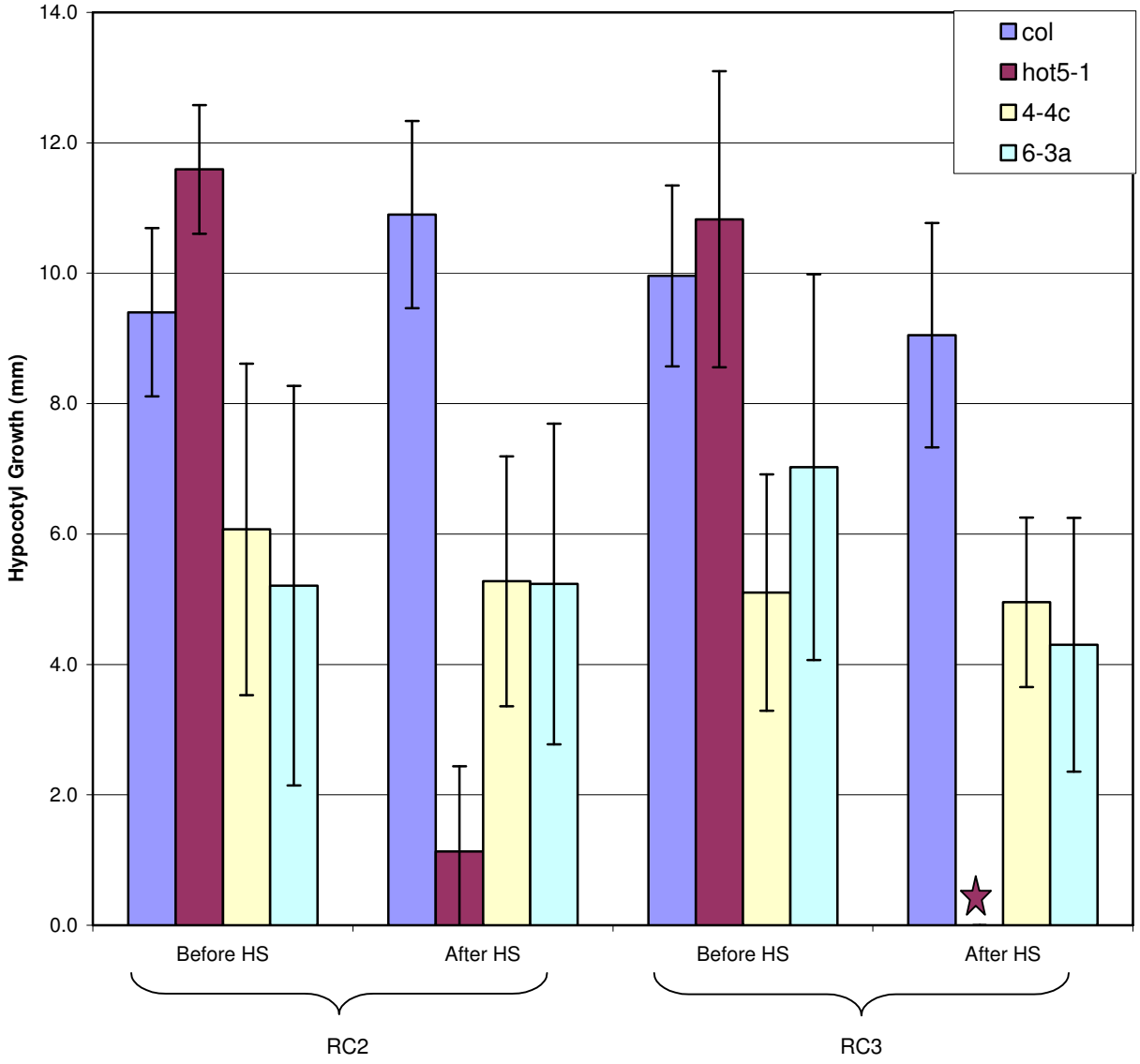


Figure 7: Average hypocotyl growth before and after heat stress for controls and three reconfirmed suppressor mutants. In each trial, 24 seeds were planted for each suppressor mutant and 6 seeds planted for both wild-type and *hot5-1*. 2.5-day-old plants were heat shocked at 38°C for 90 minutes, 22°C for 2 hours, and 45°C for 2 hours. The before heat stress (HS) data was collected directly after treatment, and after HS data was collected 2.5 days after treatment. Three trials are shown (RC5, RC6 and RC7). The error bars represent standard deviations of hypocotyl elongation measurements.

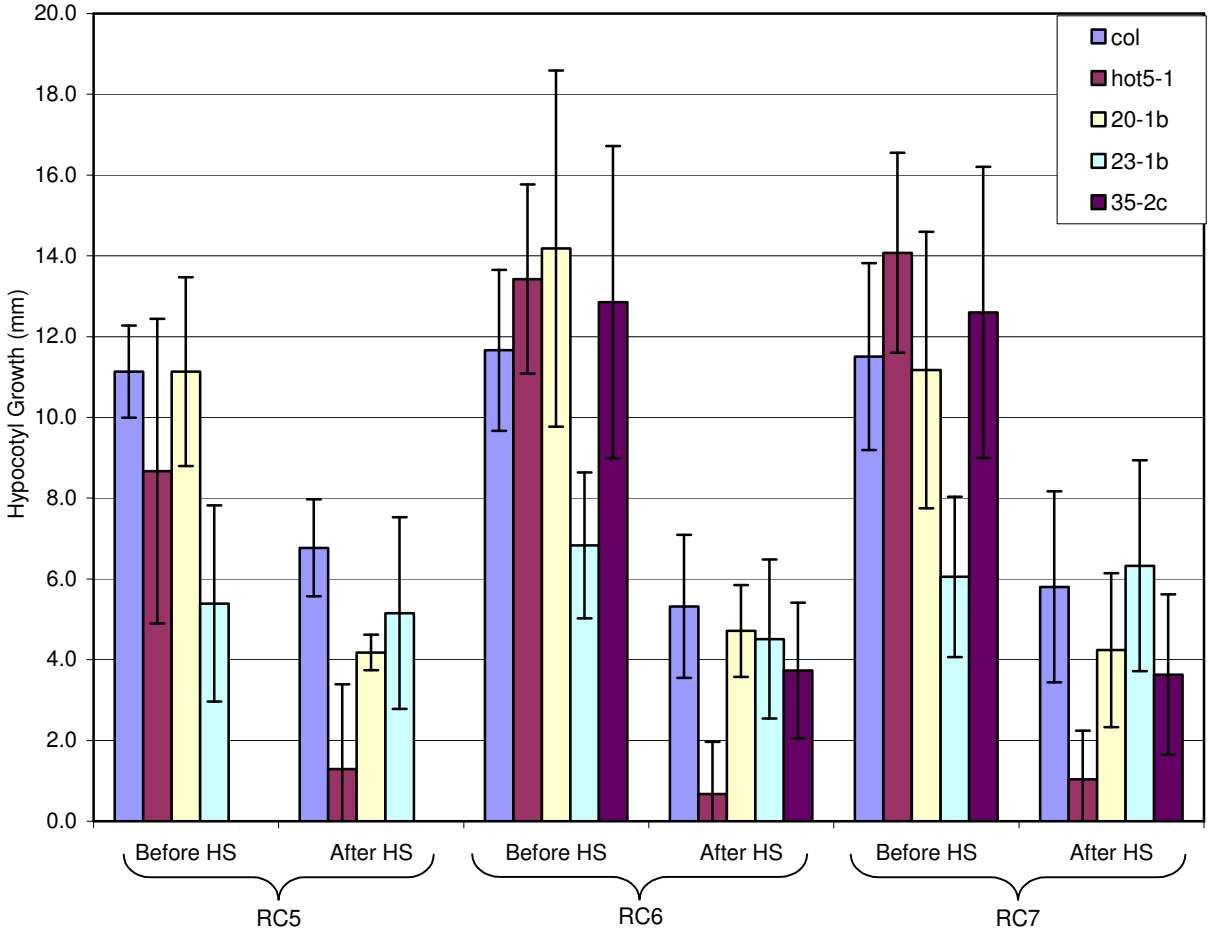


Figure 8: Sequencing chromatograms of *hot5-1* mutation region of suppressor mutants compared with wild-type and *hot5-1* sequences. The *hot5-1* mutation is highlighted with a gray box. As shown, the suppressor mutant sequences match the *hot5-1* mutant sequence within the *HOT5* region.

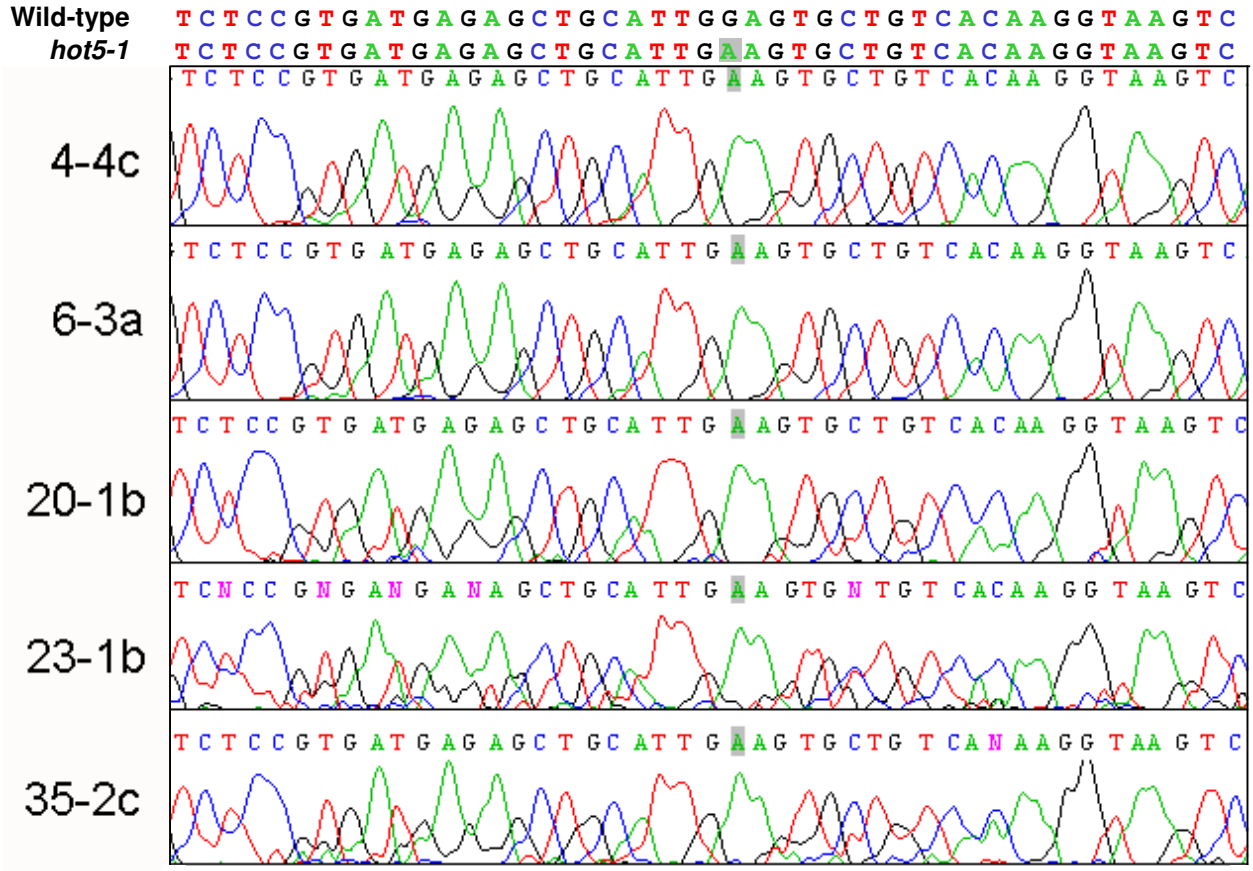
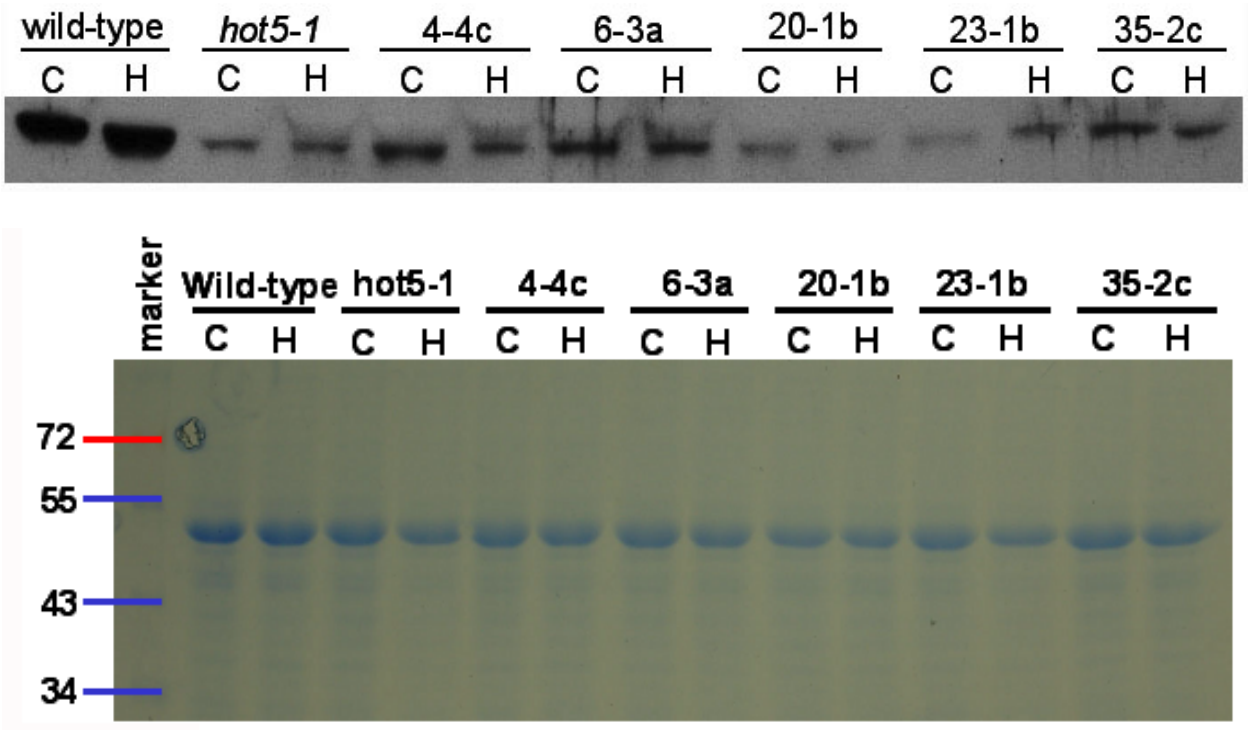


Figure 9: Western blot analysis of confirmed suppressor mutants. Samples were prepared from 25-day-old seedlings that were incubated at 38°C for 2 hours and isolated after an additional 2 hours at 22°C. Protein levels were detected using a rabbit antiserum against Arabidopsis GSNOR. Below, a Coomassie stained gel is shown to indicate amount of protein loaded in each lane. Even with the slight variation of protein amounts loaded, all five suppressor mutants have GSNOR protein expression at levels considerably less than wild-type.



APPENDICES

Appendix 1: Average hypocotyl growth for suppressor mutants reconfirmed in the M3 generation. Hypocotyl growth for wild-type and *hot5-1* after heat stress is given as a control for comparison. 2.5-day-old plants were heat shocked at 38°C for 90 min, 22°C for 2 hrs, and 45°C for 2 hrs. The before heat stress (HS) data was collected directly after treatment, and after HS data was collected 2.5 days after treatment. Multiple trials are shown, as indicated by the "RC" numbers. Data are representative of 24 suppressor seedlings, 6 or 9 *hot5-1* seedlings and 6 or 9 wild-type seedlings.

4-4c	**Before HS (mm)**	**After HS (mm)**	**Wild-type After HS (mm)**	***hot5-1* After HS (mm)**
RC2	6.1 ± 2.5	5.3 ± 1.9	11.9 ± 0.9	1.5 ± 1.3
RC3	5.1 ± 1.8	5.0 ± 1.3	9.5 ± 1.3	0.0 ± 0.0

6-3a	**Before HS (mm)**	**After HS (mm)**	**Wild-type After HS (mm)**	***hot5-1* After HS (mm)**
RC2	5.2 ± 3.1	5.2 ± 2.5	10.0 ± 1.2	0.7 ± 1.1
RC3	7.0 ± 3.0	4.3 ± 1.9	8.6 ± 2.1	0.0 ± 0.0

20-1b	**Before HS (mm)**	**After HS (mm)**	**Wild-type After HS (mm)**	***hot5-1* After HS (mm)**
RC5	11.1 ± 2.3	4.2 ± 0.4	6.3 ± 1.0	0.3 ± 0.6
RC6	14.2 ± 4.4	4.7 ± 1.1	5.6 ± 1.3	0.9 ± 1.7
RC7	11.2 ± 3.4	4.2 ± 1.9	6.3 ± 1.5	1.2 ± 1.5

23-1b	**Before HS (mm)**	**After HS (mm)**	**Wild-type After HS (mm)**	***hot5-1* After HS (mm)**
RC5	5.4 ± 2.4	5.2 ± 2.4	7.3 ± 1.3	2.0 ± 2.5
RC6	6.8 ± 1.8	4.5 ± 2.0	5.1 ± 2.1	1.0 ± 1.3
RC7	6.1 ± 2.0	6.3 ± 2.6	4.8 ± 1.9	1.4 ± 1.1

35-2c	**Before HS (mm)**	**After HS (mm)**	**Wild-type After HS (mm)**	***hot5-1* After HS (mm)**
RC6	12.9 ± 3.9	3.7 ± 1.7	5.4 ± 1.9	0.0 ± 0.0
RC7	12.6 ± 3.6	3.6 ± 2.0	6.4 ± 3.4	0.4 ± 0.9

Appendix 2: Raw data of before heat stress (BHS) and after heat stress (AHS) hypocotyl elongation for each of the five confirmed suppressor mutants. Multiple trials are shown and are labeled as "RC#."

4-4c:

RC2	**BHS**	**AHS**
4-4c	5.4	4.5
	8.1	6.3
	5.4	2.4
	2.2	3.5
	4.2	5.6
	7.7	7.6
	8.1	5.0
	6.2	2.9
	8.2	0.0
	7.6	4.1
	4.8	7.5
	6.9	7.1
	1.9	5.7
	5.7	7.5
	10.9	5.6
	7.6	5.7
	8.0	5.0
	0.0	5.7
	7.3	6.3
	5.8	7.3
	7.9	4.2
	8.2	3.2
	5.5	7.5
	2.1	6.4
Average	**6.1**	**5.3**
Standard Deviation	**2.5**	**1.9**

hot5-1	**BHS**	**AHS**
	12.1	0.0
	12.3	0.0
	12.6	1.6
	11.6	3.1
	10.9	2.9
	12.3	1.6
Average	**12.0**	**1.5**
Standard Deviation	**0.6**	**1.3**

Wild-type	**BHS**	**AHS**
	9.4	11.7
	11.2	12.4
	9.8	11.1
	9.6	12.1
	9.4	13.2
	10.8	10.9
Average	**10.0**	**11.9**
Standard Deviation	**0.8**	**0.9**

RC3	BHS	AHS
4-4c	6.4	4.2
	3.3	6.9
	3.1	7.2
	6.8	4.9
	9.5	3.6
	4.5	5.6
	5.4	4.9
	4.0	6.2
	4.8	3.6
	5.0	5.5
	4.8	4.8
	1.9	2.6
	6.9	3.8
	8.4	2.9
	3.2	5.0
	3.5	3.8
	5.2	4.7
	3.1	6.1
	6.3	4.6
	5.8	5.1
	3.2	7.3
	5.4	3.6
	6.6	5.9
	5.4	6.1
Average	**5.1**	**5.0**
Standard Deviation	**1.8**	**1.3**

Wild-type	BHS	AHS
	10.5	9.6
	10.5	10.2
	6.1	7.3
	10.1	9.0
	9.2	10.2
	9.7	10.9
Average	**9.4**	**9.5**
Standard Deviation	**1.7**	**1.3**

hot5-1	**BHS**	**AHS**
	10.4	0.0
	5.9	0.0
	10.7	0.0
	12.5	0.0
	9.9	0.0
	11.2	0.0
Average	**10.1**	**0.0**
Standard Deviation	**2.2**	**0.0**

6-3a:

RC2	BHS	AHS
6-3a	4.2	4.9
	9.9	0.0
	3.4	5.5
	3.2	6.8
	3.2	10.1
	7.6	6.4
	4.3	6.5
	8.3	6.6
	3.3	6.9
	10.9	5.3
	6.8	5.4
	1.2	2.0
	2.9	3.8
	1.3	2.1
	7.6	6.2
Average	**5.2**	**5.2**
Standard Deviation	**3.1**	**2.5**

Wild-type	BHS	AHS
	9.6	10.7
	5.9	8.1
	9.8	10.9
	9.1	11.2
	8.9	8.9
	9.8	9.9
Average	**8.9**	**10.0**
Standard Deviation	**1.5**	**1.2**

hot5-1	**BHS**	**AHS**
	12.6	1.4
	11.0	0.0
	11.7	0.0
	10.0	3.0
	9.7	0.0
	12.3	0.0
Average	**11.2**	**0.7**
Standard Deviation	**1.2**	**1.2**

RC3	BHS	AHS
6-3a	12.3	4.4
	2.1	3.0
	10.9	5.4
	9.7	3.3
	3.7	6.6
	6.8	2.2
	5.0	5.7
	6.9	5.9
	8.1	4.9
	7.9	3.8
	5.5	6.4
	5.4	0.0
Average	**7.0**	**4.3**
Standard Deviation	**3.0**	**1.9**

Wild-type	BHS	AHS
	10.2	9.8
	11.7	5.8
	10.3	10.2
	9.5	10.2
	10.9	9.4
	10.8	6.0
Average	**10.6**	**8.6**
Standard Deviation	**0.7**	**2.1**

hot5-1	**BHS**	**AHS**
	12.1	0.0
	11.6	0.0
	8.6	0.0
	14.8	0.0
	11.4	0.0
Average	**11.7**	**0.0**
Standard Deviation	**2.2**	**0.0**

20-1b:

RC5	BHS	AHS
20-1b	11.1	4.3
	14.2	4.3
	11.9	3.8
	7.6	4.1
	12.8	4.3
	13.6	3.8
	7.7	3.6
	10.6	4.3
	10.7	5.1
Average	**11.1**	**4.2**
Standard Deviation	**2.3**	**0.4**

Wild-type	BHS	AHS
	10.6	7.2
	10.4	7.3
	13.4	5.6
	9.6	6.2
	12.1	5.1
Average	**11.2**	**6.3**
Standard Deviation	**1.5**	**1.0**

hot5-1	**BHS**	**AHS**
	10.1	0.0
	13.1	0.0
	13.4	0.0
	7.7	1.2
Average	**11.1**	**0.3**
Standard Deviation	**2.7**	**0.6**

RC6	BHS	AHS
20-1b	15.4	5.7
	18.2	4.3
	13.5	3.5
	17.0	5.5
	15.7	5.3
	9.5	1.8
	16.9	5.3
	19.8	4.1
	7.9	6.3
	17.9	5.7
	13.4	3.6
	18.3	4.5
	11.8	4.7
	13.5	5.3
	3.9	5.1
Average	**14.2**	**4.7**
Standard Deviation	**4.4**	**1.1**

Wild-type	BHS	AHS
	12.3	5.5
	11.3	5.9
	8.7	7.9
	8.6	3.9
	11.8	6.1
	8.5	5.5
	12.0	4.1
Average	**10.5**	**5.6**
Standard Deviation	**1.8**	**1.3**

hot5-1	**BHS**	**AHS**
	13.9	0.8
	14.8	3.0
	9.9	4.6
	13.2	0.0
	14.5	0.0
	12.1	0.0
	14.6	0.0
	11.9	0.0
	16.6	0.0
Average	**13.5**	**0.9**
Standard Deviation	**2.0**	**1.7**

RC7	BHS	AHS
20-1b	14.9	2.5
	12.4	4.7
	8.0	0.0
	6.6	0.0
	14.3	5.7
	9.2	6.1
	8.5	3.7
	13.3	4.7
	14.9	5.5
	11.9	5.7
	15.4	4.4
	14.2	6.2
	5.3	3.9
	12.1	4.5
	11.8	5.1
	6.0	5.1
Average	**11.2**	**4.2**
Standard Deviation	**3.4**	**1.9**

Wild-type	BHS	AHS
	13.5	4.3
	9.5	7.7
	9.9	7.9
	13.7	4.0
	11.6	5.6
	12.1	7.4
	9.2	7.7
	12.6	5.4
	10.3	6.4
Average	**11.4**	**6.3**
Standard Deviation	**1.7**	**1.5**

hot5-1	BHS	AHS
	15.1	1.1
	15.3	1.4
	7.7	3.4
	17.6	0.0
	13.4	3.3
	10.2	0.0
	13.3	0.0
	14.5	0.0
Average	**13.4**	**1.2**
Standard Deviation	**3.1**	**1.5**

23-1b:

RC5	BHS	AHS
23-1b	7.0	1.7
	11.1	2.6
	2.3	5.8
	5.7	2.9
	5.4	7.9
	2.5	6.6
	6.2	6.3
	6.3	5.9
	7.4	6.5
	4.3	6.7
	4.7	8.2
	2.3	3.4
	4.0	4.3
	5.6	5.7
	3.4	8.8
	6.6	3.4
	3.7	7.2
	3.7	0.0
	10.2	4.0
Average	**5.4**	**5.2**
Standard Deviation	**2.4**	**2.4**

Wild-type	BHS	AHS
	11.2	6.7
	10.3	7.9
	10.8	6.6
	12.3	5.9
	10.6	9.2
Average	**11.0**	**7.3**
Standard Deviation	**0.8**	**1.3**

hot5-1	**BHS**	**AHS**
	11.1	0.0
	7.6	0.0
	5.3	0.0
	11.0	2.5
	5.9	2.8
	1.5	6.4
Average	**7.1**	**2.0**
Standard Deviation	**3.7**	**2.5**

RC6	BHS	AHS
23-1b	7.0	2.7
	7.2	2.5
	7.8	6.2
	10.6	0.0
	9.0	5.4
	8.2	5.8
	6.2	2.9
	5.7	6.6
	4.3	5.0
	5.5	7.8
	4.0	5.2
	4.5	5.1
	6.7	3.6
	7.8	4.5
	6.4	6.1
	8.4	2.8
Average	**6.8**	**4.5**
Standard Deviation	**1.8**	**2.0**

Wild-type	BHS	AHS
	13.0	2.8
	8.4	8.4
	15.9	4.3
	14.4	5.0
	11.1	4.3
	13.3	4.8
	10.7	7.6
	11.9	6.4
	14.3	2.1
Average	**12.6**	**5.1**
Standard Deviation	**2.3**	**2.1**

hot5-1	**BHS**	**AHS**
	16.3	0.0
	15.0	0.0
	8.2	0.0
	16.3	0.0
	7.6	0.0
	12.5	1.4
	15.4	2.6
	12.5	3.3
	14.4	1.8
Average	**13.1**	**1.0**
Standard Deviation	**3.3**	**1.3**

RC7	BHS	AHS
23-1b	6.9	7.7
	6.4	7.1
	9.2	4.6
	4.4	8.1
	5.9	5.3
	2.9	2.3
	9.6	6.6
	4.7	2.0
	4.3	10.7
	5.6	5.3
	5.8	8.1
	5.5	8.0
	6.1	3.6
	7.1	6.5
	3.2	9.3
	3.6	10.8
	7.9	7.0
	6.6	3.8
	9.3	3.4
Average	**6.1**	**6.3**
Standard Deviation	**2.0**	**2.6**

Wild-type	BHS	AHS
	9.3	6.0
	13.1	4.3
	8.9	8.3
	12.7	5.4
	14.1	4.0
	10.8	3.6
	12.2	6.4
	14.3	2.3
	13.2	3.0
Average	**12.1**	**4.8**
Standard Deviation	**2.0**	**1.9**

hot5-1	BHS	AHS
	16.3	0.0
	12.1	0.0
	14.4	0.0
	15.2	1.8
	12.8	1.5
	12.4	2.2
	17.0	3.0
	12.0	2.4
	15.3	1.3
Average	**14.2**	**1.4**
Standard Deviation	**1.9**	**1.1**

35-2c:

RC6	BHS	AHS
35-2c	15.7	4.8
	11.5	3.1
	17.0	5.2
	14.0	4.0
	13.3	4.0
	3.6	3.3
	12.9	6.7
	13.8	5.7
	13.6	3.1
	17.5	5.6
	17.5	0.0
	18.3	3.8
	4.4	2.7
	12.6	2.7
	9.4	0.0
	15.8	4.4
	14.1	3.4
	8.8	3.3
	11.2	4.2
	12.9	6.2
	14.9	2.6
	10.0	3.3
Average	**12.9**	**3.7**
Standard Deviation	**3.9**	**1.7**

Wild-type	BHS	AHS
	13.5	2.4
	13.0	4.9
	11.0	5.6
	11.4	7.3
	12.7	6.5
	9.2	6.7
	12.1	2.7
	10.8	7.0
Average	**11.7**	**5.4**
Standard Deviation	**1.4**	**1.9**

hot5-1	**BHS**	**AHS**
	16.3	0.0
	13.4	0.0
	11.8	0.0
	11.9	0.0
	12.3	0.0
	14.6	0.0
	13.9	0.0
	15.2	0.0
Average	**13.7**	**0.0**
Standard Deviation	**1.6**	**0.0**

RC7	BHS	AHS
35-2c	14.9	6.5
	14.5	0.0
	13.8	5.9
	13.3	2.3
	11.2	2.5
	3.6	5.8
	18.5	0.0
	11.1	3.7
	9.6	3.8
	14.2	4.4
	5.6	6.6
	12.1	2.0
	11.6	2.5
	13.4	1.6
	17.1	3.8
	12.3	4.9
	14.3	3.9
	11.6	5.6
	16.7	3.2
Average	**12.6**	**3.6**
Standard Deviation	**3.6**	**2.0**

Wild-type	BHS	AHS
	14.7	4.7
	9.6	9.2
	13.1	3.8
	6.7	10.1
	14.7	3.8
	6.4	10.9
	11.9	1.8
	11.1	6.9
Average	**11.0**	**6.4**
Standard Deviation	**3.3**	**3.4**

hot5-1	**BHS**	**AHS**
	12.2	0.0
	12.9	0.0
	11.5	0.0
	16.7	0.0
	13.2	0.0
	13.8	0.0
	17.3	2.4
	17.8	1.1
Average	**14.4**	**0.4**
Standard Deviation	**2.5**	**0.9**