Role of GSNOR in maintaining NO homeostasis and effect of S-nitrosation of GSNOR cysteine residues in phenotypes of *Arabidopsis thaliana*

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Abstract

Nitric Oxide (NO) is a reactive oxygen species that acts as a signaling molecule in regulating activities during plant growth, development and immune function, and it can also cause cellular damage in producing reactive nitrogen species. Therefore, control of NO homeostasis is important to plants, as well as other organisms. NO is present in cells as an adduct with glutathione (GSH), forming S-nitrosoglutathione (GSNO). S-nitrosation is a post-translational modification where a transfer of the NO moiety from GSNO to the reactive cysteine thiol group on proteins takes places, creating "SNO" protein species. S-Nitrosoglutathione Reductase (GSNOR) is known to regulate SNO levels in cells by catalyzing the reduction of GSNO, ultimately producing GSSG and $NH^{4+.}$ In GSNOR from *Arabidopsis thaliana* solvent-accessible and non-zinc-chelating cysteine residues, C-10, C-271, and C-370, are targets of S-nitrosation and their nitrosation has been shown to affect GSNOR activity *in vitro*. This thesis discusses overall growth and development of transgenic *A. thaliana* lines where C-10, C-271, and C-370 residues of GSNOR have been individually replaced with alanine, which cannot be nitrosated, in the background of a GSNOR null mutant (*hot5-2*). Western blot analysis, phenotypic assays under optimal growth conditions, and stress tolerance assays were performed to compare the transgenic lines to WT-Col and *hot5-2* GSNOR null mutant. Western blot analysis showed that all transgenic lines, similar to the WT, expressed the introduced GSNOR transgene. Non-stress phenotypes included observing plants using a GoPro camera, measuring primary root growth, lateral root growth, silique length, and observing trichomes. All of these assays demonstrated that the transgenic lines have phenotypes similar to WT suggesting that nitrosation of these cysteine residues was not required for growth in the absence of stress. Heat stress and salt stress assays were also performed, but further trials need to be conducted to have conclusive results.

Collectively, this thesis attempts to understand the difference in phenotypes and plant development when GSNOR cysteine residues that are nitrosated in vitro are replaced with alanine, testing the importance of GSNOR nitrosation to regulation of this enzyme.

Acknowledgements

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List of Abbreviations

NO: Nitric Oxide

GSNO: S-Nitrosoglutathione

GSNOR: S-nitrosoglutathione Reductase

WT: Wild Type, *Arabidopsis thaliana*, Col-0 background

HOT5: GSNOR gene (At5g43940), isolated as a mutant sensitive to hot temperatures

hot5-2: T-DNA insertion obtained from the GABI (German Plant Genomics Program; 315D11). a protein null for GSNOR

C10A: Cysteine-10 residue of *Arabidopsis thaliana* GSNOR mutated to Alanine

C271A: Cysteine-271 residue of *Arabidopsis thaliana* GSNOR mutated to Alanine

C370A: Cysteine-370 residue of *Arabidopsis thaliana* GSNOR mutated to Alanine

1. Introduction

Arabidopsis thaliana is a small flowering plant that has a life cycle of approximately six weeks. The short generation time, small size, ability to self-pollinate, small genome, and the ease of creating mutagenized populations make it relevant for plant genetic research (Gepstein and Horwitz 1995). The genome of *A. thaliana* was the first plant genome to be completely sequenced and was published in 2000 (Arabidopsis Genome Initiative 2000). My project involves using this model plant to test the significance and function of nitric oxide (NO) in plant growth, development and response to stress.

1.1 Role of NO in plants

NO has multiple roles in plants that include affecting seed dormancy, germination, fertility, stomatal closure, and pathogen resistance (Boudoin et al., 2014). NO plays a regulatory role in *A. thaliana* and other plants, controlling aspects of plant growth, maturation, and response to stress factors that hinder growth. NO is a free radical, which means it can react with metals and other free radical oxygen species. It can affect the function, activity, stability, and localization of target proteins by posttranslational modifying specific cysteine residues (León 2016). NO also has an effect on seed germination, response to external abiotic stresses, fertility and root growth, all of which were observed as part of this research.

1.2 Synthesis and mechanisms of NO action

There are two pathways for the formation of NO. One is the reduction of NO_3^- by nitrate reductase, which is an enzyme localized in the cytosol. The other proposed pathway is conversion of L-arginine to NO by NO synthetase (NOS) (Figure 1A). Plants lack NOS, but it is

assumed that the same activity can be carried out by multiple enzymes in plants that result in the production of NO from L-arginine (Jahnová et al., 2019). Due to its short half-life, the bioactive form of NO is formed by reaction with glutathione (GSH), generating S-nitrosoglutathione (GSNO), which is responsible for storing and transporting NO throughout the cell (Guistarini et al., 2019) (Figure 1B). The level of GSNO is regulated by the enzyme S-Nitrosoglutathione Reductase (GSNOR). GSNOR is a dimeric type III alcohol dehydrogenase that is crucial to NO homeostasis (Jensen et al., 1998). It catalyzes the reduction of GSNO using NADH that results in the unstable intermediate N-hydroxysulfinamide (GSNHOH). In areas of high concentration of GSH, GSNHOH is converted to glutathione disulfide (GSSG) and hydroxylamine (Figure 1C). In areas of low GSH concentration, GSNHOH is spontaneously converted to glutathione sulfinamide ($GSONH_2$) that can be converted to glutathione sulfinic acid (GSOOH) and ammonia (Jahnová et al., 2019).

1.3 Role of GSNOR in NO Homeostasis

GSNOR (*AtGSNOR1*) irreversibly degrades GSNO in plants, which reduces the S-nitrosation activity of GSNO that is crucial for maintaining NO homeostasis (Zhan, et al., 2018). NO and GSH form GSNO, which is the cellular NO reservoir that is involved in NO signaling. GSNOR metabolizes GSNO, and in contrast, GSNO is able to nitrosate GSNOR to reduce its activity. The model for the role of GSNOR in controlling NO signaling is depicted in Figure 1D. When GSNO levels increase due to production of NO due to intra-cellular or extracellular changes, GSNOR would be nitrosated, decreasing its activity. This would decrease GSNO breakdown, effectively increasing the NO signal. As GSNOR is gradually denitrosated, and reactivated, the GSNO would be metabolized, returning NO to normal levels. My experiments involve studies of mutants of GSNOR in *A. thaliana* in order to examine possible mechanisms regulating GSNOR

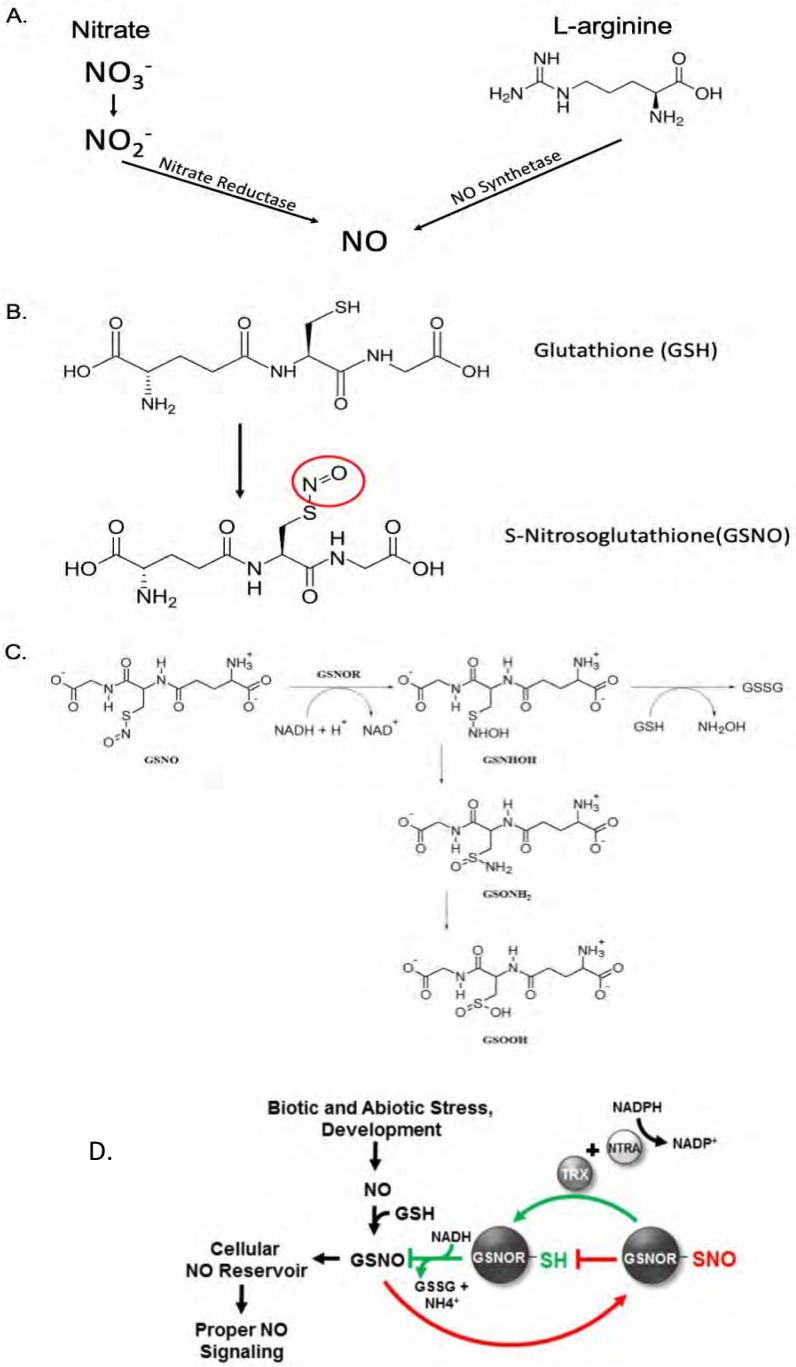

Figure 1. Synthesis of NO and S-nitrosoglutathione (GSNO), and the reduction of GSNO

(A) Pathways of formation of NO.
(B) Formation of S-nitrosoglutathione (GSNO) from Glutathione (GSH)
(C) Reduction of GSNO by GSNOR where NADH is used as a proton donor (Adapted from Jahnová et al., 2019).

(D) Model for regulation of GSNOR and its involvement in NO homeostasis (Adapted from Vierling Lab)

activity. GSNOR contains evolutionarily conserved cysteines (Cys-10, Cys-271 and Cys-370) that could serve as nitrosation sites and act to control GSNOR activity, as proposed from in vitro experiments (Guerra et al., 2016). Among all cysteine residues in GSNOR, C10, C271, and C370 are present on the surface of the dimer, which allows them to be solvent accessible sites for nitrosation (Xu et al., 2008). The structure of the GSNOR monomer is shown in Figure 2A where C10 and C370 are on the outer region of the protein and C271 is closer to the active site and dimer interface.

To determine if C10, C271, and C370 could be nitrosated, Guerra et al. (2016) developed cysteine-to-alanine mutants in which each of the residues were replaced with alanine. C177 is a zinc-chelating residue in the enzyme active site, and it was used to as a control that would lack activity, even though it would not be nitrosated. The purified, recombinant GSNOR WT and mutants were treated with the NO donor CysNO and the change in protein mass was examined by mass spectroscopy. For the WT proteins, four mass species were observed; the peak at 42726.8 daltons is the WT reduced peak, and the other three peaks represent mono-nitrosated, di-nitrosated, and tri-nitrosated peaks (Figure 2B). Each peak has a difference of 30 daltons, which is the molecular mass of nitric oxide, suggesting that three residues had been nitrosated. When the C10, C271, and C370 residues are replaced with alanine, there can be no nitrosation of the residue. Thus, there are only two peaks suggesting that only two of the three residues were nitrosated meaning that the two cysteine residues except for the alanine were nitrosated (Figures 2D-G). The presence of all three peaks when the zinc-chelating residue C177 is replaced with alanine confirms that C177 is not a target of nitrosation (Figure 2E). Finally, when all three of the cysteine residues are replaced with alanine, no additional peaks were observed. Thus, this study confirmed that C10, C271, and C370 residues are targets for S-nitrosation *in vitro*.

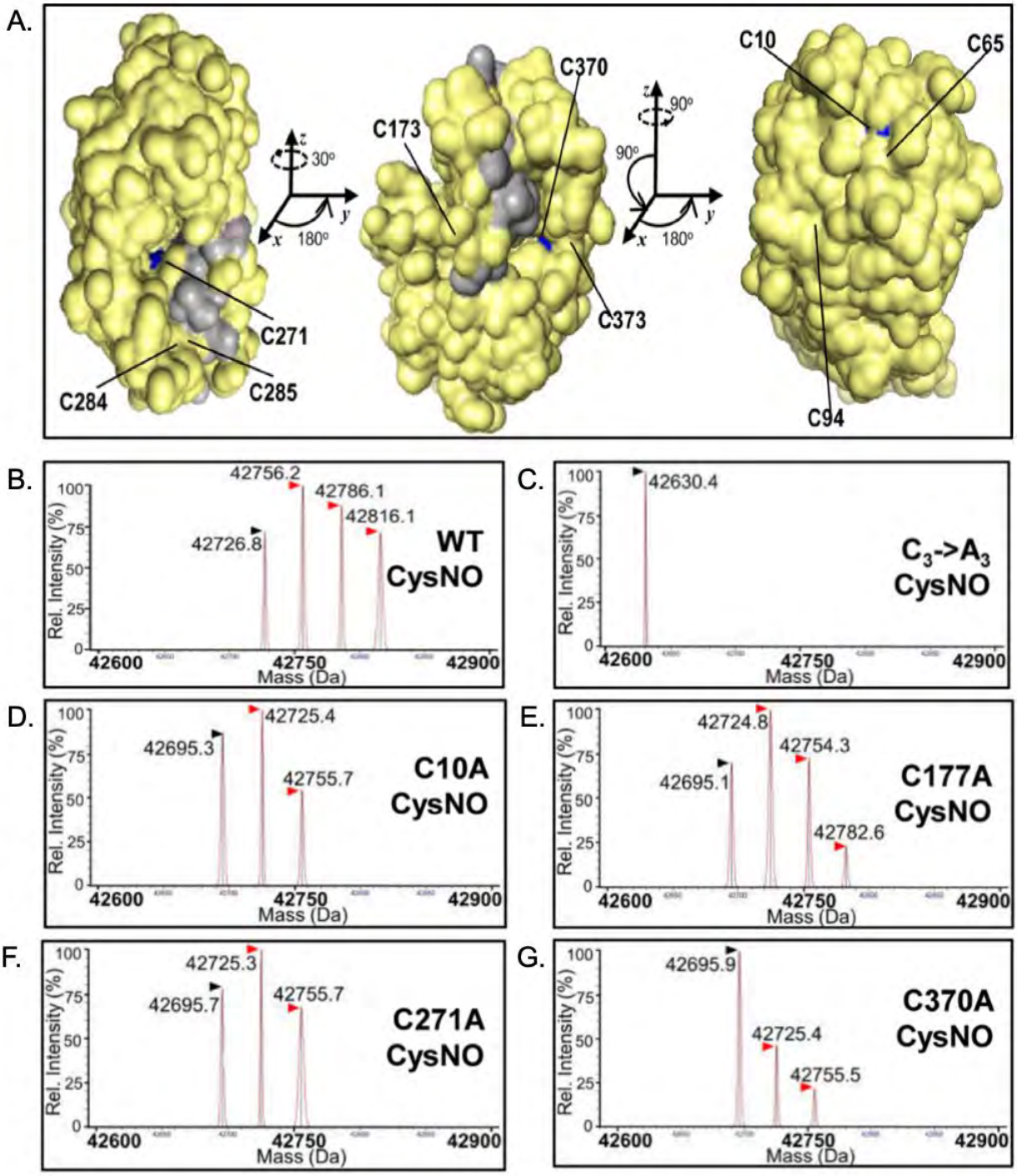

Figure 2. GSNOR Dimer and mass spectrometry of WT and Cys-Ala mutants of GSNOR
(A) The structure of the GSNOR monomer. Yellow represents the solvent accessible residues, gray represents the dimer interface, and blue represents the solvent accessible cysteine residues. (B-G) Mass spectroscopy data of wild-type and mutants to show the nitrosation of cysteine

residues in GSNOR. Black arrow points to the peak of unmodified GSNOR, and red arrows represent nitrosated GSNOR adducts with a difference of 29 daltons between each peak. (A) is adapted from Xu et al. (2013) and (B-G) are adapted from Guerra et al. (2016).

GSNOR was shown to be sensitive to inhibition by nitrosation (Guerra et al., 2016). When unmodified GSNOR was treated with NO donors GSNO and SNAP, the activity of GSNOR decreased over time. The activity of GSNOR is proposed to be regulated by post-translational modifications, including S-nitrosation and phosphorylation. S-nitrosation is a post-translational modification that covalently attaches a NO group to a cysteine to form S-nitrosothiol (SNO) (Guerra, et al., 2016). For my research, *A. thaliana* plants HOT5 were used. A T-DNA insertion allele *hot5-2* was obtained and was backcrossed to WT-Col (Lee et al., 2008). The *hot5-2* mutant that is null for the *GSNOR* gene (AT5G43940) have been "complemented" by transformation with *GSNOR* genes in which either the Cys-10, Cys-271 or Cys-370 residues have been replaced by Ala to test their significance to the function of the protein and impact on plant phenotype.

1.4 Effects of NO in plant development

A. thaliana WT and *hot5-2* plants have visible differences in leaves, root, branches, and overall growth. *hot5-2* mutants have a multi-branching shoot phenotype, which means the apical meristem is able to grow multiple shoots (Lee, et al., 2008). Compared to the WT plant, *hot5-2* morphology is shorter with more branches (Figure 3A). Branching phenotypes are controlled by auxin and cytokinin pathways in shoots, and it is suggested that GSNOR regulates a step downstream that affects shoot branching (Kwon, et al., 2012), but the precise mechanism is still unknown.

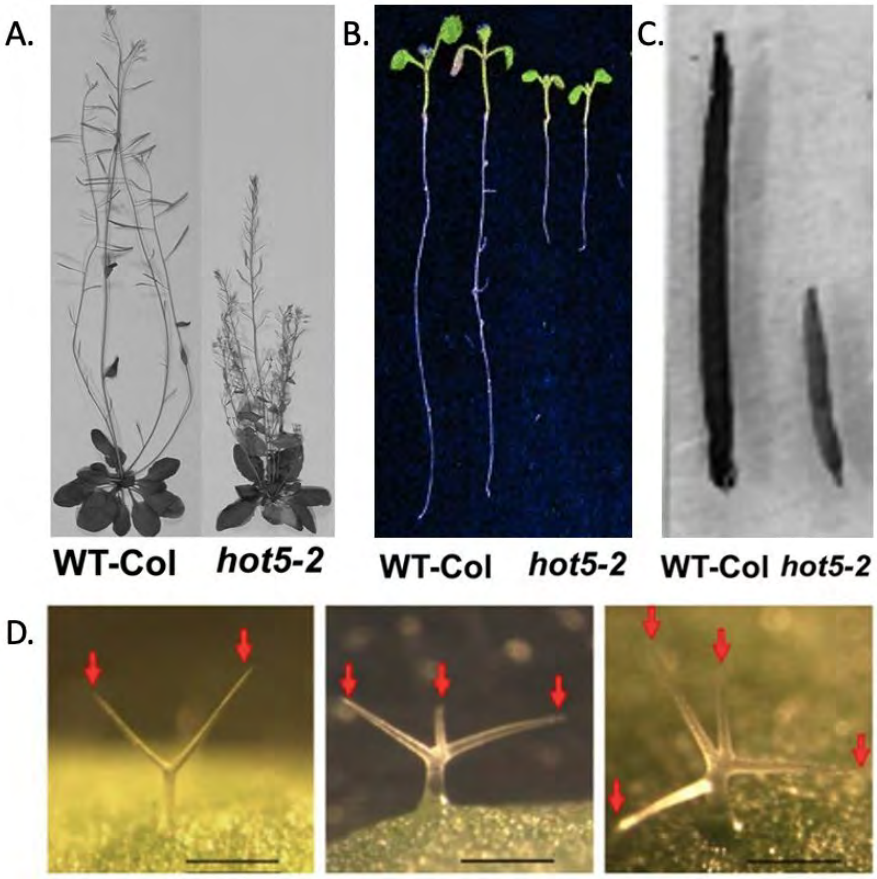

Figure 3. Phenotypic differences between WT and *hot5-2*.

(A) Comparison of 45-day old, soil-grown, WT and *hot5-2* plants (Adapted from Lee et al., 2008)
(B) Primary root length of WT and *hot5-2* observed in 10-day-old light-grown seedlings on 1/2 MS Agar medium (Adapted from Kwon et al., 2012)
(C) Length of full-grown siliques of WT and *hot5-2* (Adapted from Lee et al., 2008)
(D) Left to right: trichomes from *hot5-2*, WT-Col, WT-Col. Bar: 0.2mm. Red Arrows indicate individual trichome branches (Adapted from Xu et al., 2013)

GSNOR null mutants also have impeded root development and show short roots and lack lateral root development (Kwon, et. al 2012). Lateral roots extend horizontally from the primary root to anchor the plant to the soil. Wild type plants develop lateral roots early in their growth; however, the mutants do not show lateral root growth (Figure 3B).

The *hot5-2* mutants have significantly reduced fertility and produce fewer seeds compared to wild type. The mutant plants grow numerous leaves and flowers perhaps as a

response to reduced fertility, but seed set is poor (Figure 3C). The *hot5-2* plants produce very few seeds for each plant which explains why the siliques do not elongate as the wild type siliques (Lee, et al., 2008).

Leaves have trichomes on the surface, which are epidermal hairs that have a branched morphology. Trichomes serve multiple functions in plants which include protection against radiation damage, protection from insects by serving as mechanical hinderance to attackers, reduce wind velocity and lose of water, and serve as storage sites for heavy metals (Jakoby, et al., 2008). Wild-type plants produce trichomes with three or four branches whereas the *hot5-2* mutants produce trichomes with only two branches (Figure 3D). Absence of GSNOR reduces the trichome branching. 90% of *hot5-2* mutants had two-branched trichomes and 10% had three-branched trichomes, while the wild type had no trichomes with two branches, 80% three-branched trichomes, and 20% had four-branched trichomes (Xu, et al., 2013; Holzmeister, et al., 2011).

1.5 Role of NO in Heat Stress Tolerance

GSNOR activity in maintaining NO homeostasis is crucial for the plant in non-stress and under stress conditions, one of which includes acclimation to heat stress. The *hot5-2* mutant has been shown to be heat sensitive (Lee, et al., 2008). High levels of NO species in *A. thaliana hot5* mutants increases heat sensitivity as a result of the disturbance caused to sensitive pathways that involve reactive oxygen species. Under heat stress, the NO pathways in the plant are already likely to be under strain to try to maintain a proper NO-homeostasis. GSNOR has an important role in the regulation of NO, which can be related to the thermotolerance defect in *hot5-2* mutants. At high temperatures, *hot5-2* mutants lose chlorophyll and the plants do not survive

(Figure 4). The phenotypes of *hot5-2* were similar to *hot1-3,* which is a null mutant for the heat shock protein Hsp101. This suggests that lack of GSNOR activity prevents the development of heat tolerance in *A. thaliana* plants.

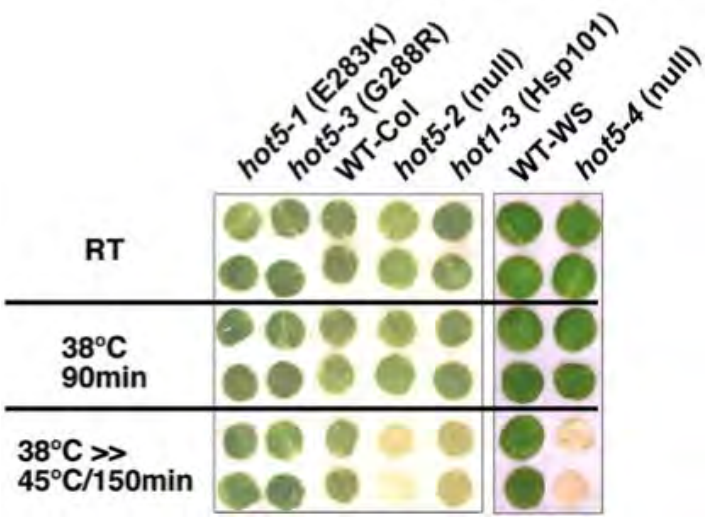

Figure 4. Thermotolerance defect in *hot5-2* plants. Heat stress acclimation of 5mm leaf discs floated on 2mL MES-KOH buffer (pH 6.8). Leaf discs were treated at room temperature (22ºC), 38ºC for 90 minutes, or 38ºC for 90 minutes followed by 2 hours at 22ºC and 150 minutes at 45ºC. Leaf discs were placed at 22ºC for 5 days and photographed after acclimation time (Adapted from Lee et al., 2008).

1.6 Role of NO in Salt Stress Tolerance

Soil salinity is a crisis and high salinity hinders normal plant growth. Higher levels of NO have been reported to allow the plant to survive salt stress, and GSNOR is reported to act as a negative regulator of salt tolerance (Zhou, et al., 2016). Depending on its concentration, NO can protect plants against salt stress by lessening the secondary oxidative stress induced by high salinity. Calmodulins (CaM) are calcium sensor proteins. Binding of calcium to CaM induces the exposure of hydrophobic clefts that can interact with downstream targets. Activation of specific CaM isoforms initiates a signaling pathway that promotes salt tolerance. There are seven genes in the *A. thaliana* genome that encode CaM, but they only code for four isoforms due to amino acid identity: AtCaM1/4, AtCaM2/3/5, AtCaM6, and AtCaM7. *Atcam3* is known to regulate the expression of genes related to cold tolerance, and *Atcam7* is involved in responses to light. *AtCaM1* and *AtCaM4* are involved in salt resistance, which is attributed to binding and inhibition

of GSNOR, which enhances NO accumulation. Results from these authors further suggest that GSNOR function is involved in salt tolerance in *A. thaliana* plants.

After exposure to salt, the expression of *AtCaM1* and *AtCam4* increased as shown in Figure 5A and Figure 5B, which suggests that those calmodulins could be involved in the resistance to salt stress. To further confirm this, Zhou et al. (2016) created CaM single knockout and double knockout lines of T-DNA insertion mutants. The single mutants *cam1-1*, *cam1-2*, and *cam4* were single mutants whose survival ratio was significantly lower that the WT plants after exposure to salt stress. The double mutants *cam1/4-1* and *cam1/4-2* had a survival ratio that was even lower than the single mutants indicating that *AtCam1* and *AtCam4* are responsible for the salt sensitivity response (Figure 5C). Another experiment was done to test the direct relation of *AtCaM1* and *AtCaM4* to NO homeostasis. NO-sensitive fluorescent dye 4-amino-5-methylamino- 29,79-difluorescein diacetate (DAF-FM DA) was used as the fluorescent probe for NO as it is highly specific for NO species and does not react with any other reactive oxygen species (Figure 5D). Fluorescence analysis revealed that the NO levels were relatively stable in seedlings under normal growth conditions. However, the NO level remarkably increased in the presence of NaCl and varied depending on the expression of *AtCaM1* and *AtCaM4*. NO levels were significantly lower in the *cam* mutants, and complementation lines had similar results to the WT.

AtCam has also been shown to inhibit GSNOR activity. Based on the graph in Figure 5E, the level of GSNOR activity in total protein extracts was greatly increased by NaCl in the *cam1-1, cam1-2, cam4, cam1/4-1*, and *cam1/4-2* mutant seedlings. Due to inhibition of GSNOR activity in the *AtCaM* transgenic lines, higher levels of NO will be present. The authors concluded that accumulation of NO assists in the development of salt tolerance in *A. thaliana*

(Zhou et al., 2016). *hot5-2* is a null mutant for GSNOR, and as *hot5-2* has been shown to have higher levels of NO, the mutant would be expected to have better salt tolerance than WT plants.

1.7 Goals of the Thesis

The hypothesis is that the enzyme GSNOR is important for regulation of NO homeostasis in *A. thaliana* and that alterations in GSNOR post-translational modification by NO at the specific mutated Cys residues may result in plants that show differential phenotypes, which would provide clues to GSNOR function and regulation during particular plant developmental stages. My experiments utilized wild-type *A. thaliana* and plants carrying the *hot5-2* null mutant allele of GSNOR that were complemented by transformation with GSNOR genes in which one of three specific Cysteine residues (Cys-10, Cys-271, and Cys-370) has been replaced by Ala. Testing for phenotypic changes of functions of the mutant plants compared to the wild type will allow us to identify the potential importance of each of the Cys residues.

Morphological phenotypes of *hot5-2* such as shorter roots, reduced fertility, multi-branching, reduced trichrome branches, and reduced later roots were observed. In addition, the reaction of mutant plants to different stresses, such as heat stress and salt stress, were tested, as the *hot5-2* mutant is more heat sensitive, but has been reported to have higher survival rates under salt stress conditions. Results provide new information about the potential significance of regulation of GSNOR by nitrosation.

2. Methods

2.1 Generation of transgenic plants

Plant transformation was performed by Dr. Patrick Treffon in the Vierling Lab. For transformation, *hot5-2* plants were grown in long day conditions until they started flowering.

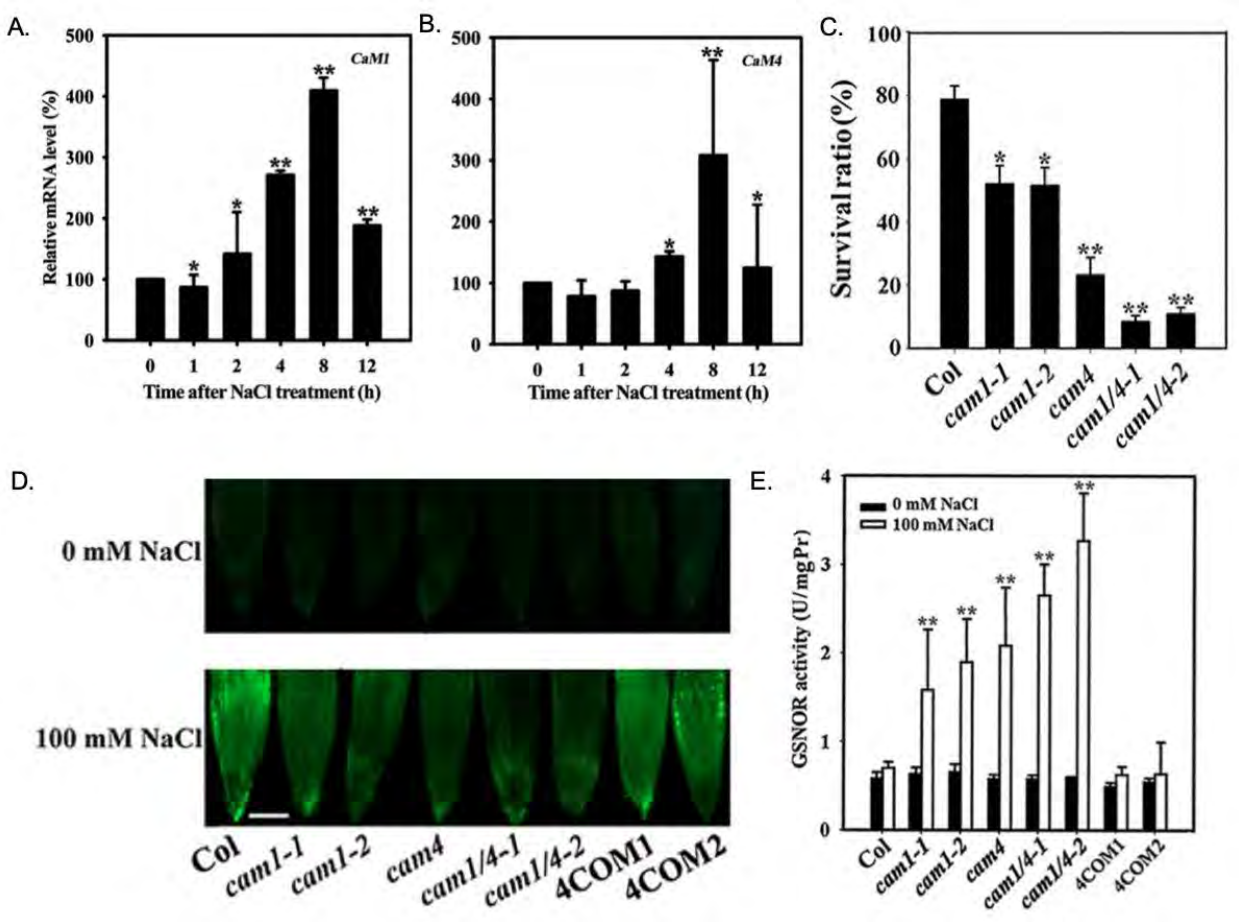

Figure 5. Salt tolerance in genotypes of *AtCaM* mutants.
(A-B) RT-qPCR analysis of *AtCaM1* and *AtCaM4* showing the relative mRNA levels of 7-day-old WT seedlings over time after treatment with 50 mM NaCl.
(C) Survival ratio in percentage of *AtCaM* mutants after growing 7-day-old seedlings in 0.5×MS medium with 100 mM NaCl.
(D) DAF-FM DA staining used to detect NO accumulation in roots of 7-day-old WT roots and *AtCaM* mutant roots grown on 0.5x MS Agar Media after 24 hours of NaCl treatment.
(E) GSNOR activity of total protein from 7-day-old WT, *cam1-1, cam1-2, cam4, cam1/4-1, cam1/4-2*, 4COM1 and 4COM2 seedlings grown in 0.5 × MS medium with or without 100 mM NaCl. Each data point represents the mean ± SD (n = 3). Asterisks indicate a significant difference relative to 0 mM NaCl (T-test, *P < 0.05 and **P < 0.01) (Adapted from Zhou et al., 2016).

Agrobacterium tumefaciens carrying plasmids were grown in 5% sucrose solution and above-ground parts of the plant were dipped into the solution for 1-3 minutes. There were 5 vectors: empty vector, WT, and three C to A mutants. The plasmid constructs contained the cysteine to alanine mutation, Basta resistance, REDSEED phenotype, and BC2 tag. The dsRED protocol was obtained from Wu et al. (2015). The REDSEED phenotype was used to determine the homozygosity of the transgenic lines depending on the level of fluorescence. It is expressed in the promoter and it causes the transformed seedlings to glow red when illuminated with green light. The dipped plants were covered in a dome and were allowed to grow in a high humidity environment until T1 seeds were mature. T1 seeds were screened by Dr. Treffon to obtain initial transgenic lines that were then allowed to self to obtain the segregating T2 generation.

2.2 Selecting homozygous transformed seeds

T2 seeds were screened to identify lines segregating 3:1 for the RED Seed marker, and individuals from those T2 lines were grown to obtain T3 seeds. From each T2 transformed plant sample, approximately 100 T3 seeds were observed under green light with red night vision glasses (600nm filter) to confirm if the seeds were homozygous for the transgene, heterozygous, or homozygous for the wild-type gene. T3 seeds from T2 plants that were assessed to be homozygous for the introduced, mutated *hot5* (GSNOR) gene were then grown to obtain a T4 stock of homozygous seeds for analysis.

2.3 Western Blot analysis

Protein extraction

Leaf samples were collected from 25-day-old plants, 100mg of plant tissue was obtained which

produced approximately 100ug of protein. Leaf samples were flash frozen in liquid nitrogen before adding 3 µL of sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 65 mM DTT, 15% sucrose, 0.01% bromophenol blue) per 1 mg of plant material. Frozen plant material was ground in the extraction buffer and samples were centrifuged for 10 minutes at maximum speed. The supernatant was transferred to a new tube for quantification.

Protein sample quantification (Bradford assay)

96-well plate was used to pipette triplicates of each sample. Protein standards containing 0, 0.2, 0.4, 0.6, and 0.8 mg/mL bovine serum albumin (BSA) and 2 µL of plant samples were spotted on filter paper and left to dry overnight. The spotted filter paper was incubated in Coomassie Stain (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) for 10 Minutes. After destaining the filter paper with H_2O, each spot was hole-punched into a tube containing 2% SDS and incubated at room temperature for 4 hours. Nanodrop Spectrophotometer was used to quantify the samples by comparing absorbances to the BSA standard curve. Coomassie Blue was used to quantify the samples and an R^2 value was calculated.

SDS Page and Membrane Transfer for Western Analysis

According to the protein quantification results, 1ug/uL dilutions of protein were added to H_2O, 5x SDS-LD and 1M DTT. Samples were loaded on a commercial gel (Novex WedgeWell 4-20% acrylamide Tris-Glycine Gel; Thermo Fisher Scientific). Gels were run to separation and blotted onto a nitrocellulose membrane for 2 hours in a semi-dry blotter. Blots were washed and incubated with Thermo Scientific SuperSignal West Femto Maximum

Sensitivity ECL Substrate. G:Box iChemi XT (Syngene) was used to visualize the blots.

Blocking and Antibody Reaction

Blots were blocked with 5% (w/v) milk in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature with gentle agitation and rinsed with TBS-T. Membranes were incubated overnight with 1:1000 dilution of the primary anti-GSNOR antibody UAZ180 or UAZ179 (generated previously with purified protein from the lab at a commercial antibody production company). Blot was rinsed briefly twice, then washed 3 times for 10 min in TBS-T at RT with agitation. Blots were incubated in 1:5000 dilution of the secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase, GE Healthcare), in TBS-T for 1 hour at room temperature with agitation. Horseradish peroxidase catalyzes the oxidation of substrates by hydrogen peroxide. This process uses the property of chemiluminescence where light is emitted as a byproduct. Luminol was used as a reagent and its oxidation by peroxide results in the excitation of a product called 3-aminophthalate. This product later emits light by releasing photons to reach a lower energy state. Blots were washed as above and incubated with Thermo Scientific SuperSignal West Femto Maximum Sensitivity ECL Substrate before visualizing with the G:Box iChemi XT (Syngene).

2.4 Non-Stress Phenotypic tests – optimal growth conditions:

Overall growth (GoPro)

Two replicates of each independent line were used for this growth assay. The plants were allowed to grow at 22°C on 12-hour day length with light intensity of 40-70 µE. A GoPro Hero7 Black was used to record plant growth. The settings for the camera were set to Protune: ON,

ISO: Min-100, Max-800 for optimal photo quality. Time lapse photo mode was set to take photos at 5-minute intervals. iMovie was used to create a movie with all the photos captured by the GoPro.

Trichome branching

To observe and analyze the trichome phenotype, 10 seedlings each of WT, *hot5-2* and *hot5-2* transgenics were grown in soil and the 30 trichomes on one leaf for each genotype was observed using a dissecting microscope.

Multi-Branching

The multi-branching phenotype of the *hot5-2* transgenics was observed with the same plants used for analysis of trichome phenotypes, with their height and branch pattern and numbers measured 8 weeks after germination.

Fertility

To observe fertility, fully grown siliques were obtained from 3 plants of each genotype of wild type, *hot5-2*, and transgenic seedlings grown on soil. Siliques were collected from the 5th to the 10th flower as the first few flowers may not develop normally. Silique length was measured using ImageJ and photographs were taken.

Lateral root growth

To observe lateral root growth, 60 seedlings of WT, *hot5-2,* and transgenic lines were grown on Murashige and Skoog (MS) Agar media with 0.5% sucrose. Seeds were sterilized to prevent bacterial or fungal growth by soaking seeds in a solution containing 49.5% Clorox and 0.5%

Tween 20 in deionized H_2O. During soaking, seeds were mixed at 22°C at 1,000 RPM for 10 minutes. The seeds were rinsed with H_2O seven times to remove traces of Clorox.

The seeds were placed on 10cm square integrid, polystyrene petri dishes containing 20 mL of Murashige and Skoog 0.8% agar media consisting of 0.5X MS Media (Sigma MO404-10L), 0.5% sucrose, and 0.8% agar. 0.5% sucrose and 0.5X MS Media powder was added to a beaker, and deionized H_2O was added. 0.25g of MES (2-(N-morpholino) ethanesulfonic acid) was added to buffer pH. While this solution was being mixed, a bottle was prepared with 0.8% (w/v) plant-based agar. The pH of the solution of sucrose and MS media was adjusted with a pH meter by the addition of 1M KOH until the final pH was 5.7. The solution was transferred to a graduated cylinder and MilliQ H_2O was added until the final volume is 250 mL, and then was sterilized and transferred to the PYREX bottle prepared with plant-based agar which was autoclaved later.

To observe any differences between root growth of the mutant and the wild type, seedlings were grown on agar plates. Under a sterile hood, 20 mL of the liquified MS agar media was poured into each of the 10 cm plates. When the media was completely solidified, n>60 sterilized seeds were plated. After placing the seeds, plates were wrapped in parafilm and the seeds were allowed to stratify in darkness at 4°C for 2 days. This was done in order to better synchronize germination. Following seed stratification, plates were placed vertically in a growth chamber at 22°C on 12-hour day length with light intensity of 40-70 μE, and growth phenotypes were analyzed. The plates were scanned daily, and root lengths were measured using ImageJ. Two-way Anova statistical test was performed in Prism and relative root length graphs were created to visualize phenotypic differences between WT, *hot5-2,* and transgenics.

2.5 Heat Stress

A leaf-disc assay was performed to compare the heat tolerance of WT, *hot5-2,* and transgenic plants. WT, *hot5-2,* and transgenic lines (Lee, et al., 2008) were planted in soil and grown at 22ºC constant for 25 days in 12-hour day length. A 5 mm disc was obtained from the third to fifth fully expanded leaf. The discs were floated in 24-well microplates containing 2mL of 10mM MES-KOH buffer with a pH of 6.8. Plates were incubated in various heat stress treatments: at 22ºC only, or 38ºC for 90 minutes followed by 22ºC for 2 hours and 45ºC for 2.5 hours. Afterwards, the leaf discs were kept at 19ºC for 5 days for the first trial and at 22ºC for the second trial. Photographs were taken to analyze the results using an LED light plate.

2.6 Salt Stress

The selected transgenic seedlings were grown on MS agar media plates for 3 days. Then, they were transferred to 12-well plates containing different concentrations of NaCl (0mM, 25mM, 50mM, 75mM, and 100mM). After 4 days under salt stress, the seedlings were placed on newly prepared MS agar media plates and photographed. The root lengths were determined using ImageJ and graphs were made to compare the growth of wild type and *hot5-2* seedlings.

Due to the difference in root development where *hot5-2* seedlings have shorter root lengths than WT, the results for the earlier protocol were inconclusive. To optimize this stress assay, *hot5-2* seedlings were allowed to grow for a longer time to get comparable root lengths of WT and *hot5-2* before performing salt stress. After 4 days of growth for WT and 8 days of growth for *hot5-2*, all seedlings were transferred to 12-well-plates with varying concentrations of NaCl and MS media (0.5X MS media, 0.5% sucrose, 0.8% agar) that include 0mM, 25mM,

50mM, 75mM, and 100mM NaCl. After 4 days of salt exposure, the seedlings were placed on MS agar plates to be photographed. Root lengths were determined as above using ImageJ.

3. Results

3.1 Transformation to create transgenic lines

Transformed plants (T1 generation) were created using the floral dip technique, which was performed by Dr. Patrick Treffon in the Vierling Lab. An *Agrobacterium tumefaciens* strain was grown with vectors of interest that would transfer the plasmid into the *A. thaliana* genome. Particularly Cys-10, Cys-271, and Cys-370 residues in the GSNOR sequence were replaced with alanine (Figure 6B-D). There were also control constructs for the transgenic plants where the GSNOR activity was mimicked in *hot5-2* and the phenotypes were expected to represent WT seedlings (Figure 6A). The transformation vector included a red seed marker (Wu, et al., 2015) to visualize the transformation, a Basta resistance gene, and GSNOR was modified with a BC2 tag (PDRKAAVSHWQQ) at the C-terminus. The BC2 tag allows the tagged protein to be pulled out of solution using affinity methods (Bruce, et al., 2017).

3.2 Isolating independent homozygous transgenic lines

Seedlings of the transgenic lines were screened to determine homozygosity and to isolate multiple independent lines for each transgene.

There were four transformation constructs: a control that reintroduced WT GSNOR, and three constructs where alanine replaced Cys-10, Cys-271, and Cys-370. All genes were controlled on their own promoters. T1 seeds were screened by shining green light (CREE LED

150 Yard Green Light Flashlight by Ulako) and observing the seedings through red filter goggles by NightSea (600nm filter), and red seeds were planted to obtain T2 seeds. Seeds from T2 plants were again screened for red fluorescence to identify plants showing a 3:1 segregation ratio for the red seed phenotype as that is the expected segregation ratio for heterozygous seedlings. The brightest red seeds were judged to be homozygous for the introduced mutant gene, heterozygous seeds were those that appeared half as bright, as they would have only one copy of the red fluorescent marker linked to the mutated gene (Figure 7).

The brightest seeds from the T2 generation were presumed to be homozygous for the transgene and were grown to obtain the T3 generation. In the T3 generation, there was varying levels of fluorescence even in the lines that were confirmed homozygotes. Brightest seeds were planted from the T3 generation to obtain T4 generation seed stocks. Three independent transgenic lines were obtained for WT and C10A constructs, and two independent lines were confirmed for the C271A and C370A constructs (Table 1).

3.3 GSNOR protein levels in the transgenic plants

Western blot analysis was performed to quantify the amount of GSNOR protein produced by each of the transgenic lines. Each of the transgenic lines have a loss-of-function mutation for the GSNOR protein, however it was expected that they have the same amount of protein expressed as wild type because the introduced GSNOR was expressed under control of the native promoter. The reversible Ponceau Stain was used to determine if equal amounts of protein were loaded for all samples. The Ponceau stain binds to the proteins producing pinkish-red bands, with the most prominent band being the Rubisco large subunit (RBCL), the most abundant plant leaf protein.

For the first blot, the UAZ180 anti-GSNOR antibody was used in a 1:1000 dilution

	Construct	Transgenic Line	Homozygosity status
WT	GSNOR WT-BC2t	13180-1	Homozygous
		13180-2	Homozygous
		13180-3	Homozygous
C10A	GSNOR C10A-BC2t	13181-1	Homozygous
		13181-2	Homozygous
		13181-3	Homozygous
C271A	GSNOR C271A-BC2t	13182-1	Homozygous
		13182-2	Homozygous
C370A	GSNOR C370A-BC2t	13183-1	Homozygous
		13183-2	Homozygous

Table 1. Status of Transgenic Lines generated through Agrobacterium-mediated transformation of *hot5-2*. The transgenic lines contain genes for Basta resistance and a BC2-tag at the C-terminus of the GSNOR gene. Each independent line used for the experiments is indicated.

followed by an anti-rabbit antibody containing horseradish peroxidase. In Figure 8A, non-specific binding is observed in all control and transgenic lines, as judged by its the presence of a reactive band in the *hot5-2* mutant sample, which is null for the GSNOR protein; the upper band is detected in WT and *hot5-2,* but not in the lane loaded with purified GSNOR (provided by Dr. Patrick Treffon). The purified GSNOR protein produces a band between 36 and 55 kDa, and it is observed in WT, but not in the *hot5-2* control, as it is a null mutant for GSNOR. Thus, the positive and negative controls were successful. No bands were seen in the sample from Transgenic line C370A-1, indicating 1 did not produce any band meaning that there was an error in protein extraction as the Ponceau stain, to confirm accurate loading, also did not have a band showing staining. All other transgenic lines produced a band in a similar location to the purified protein band, confirming the presence of GSNOR protein in transgenic lines.

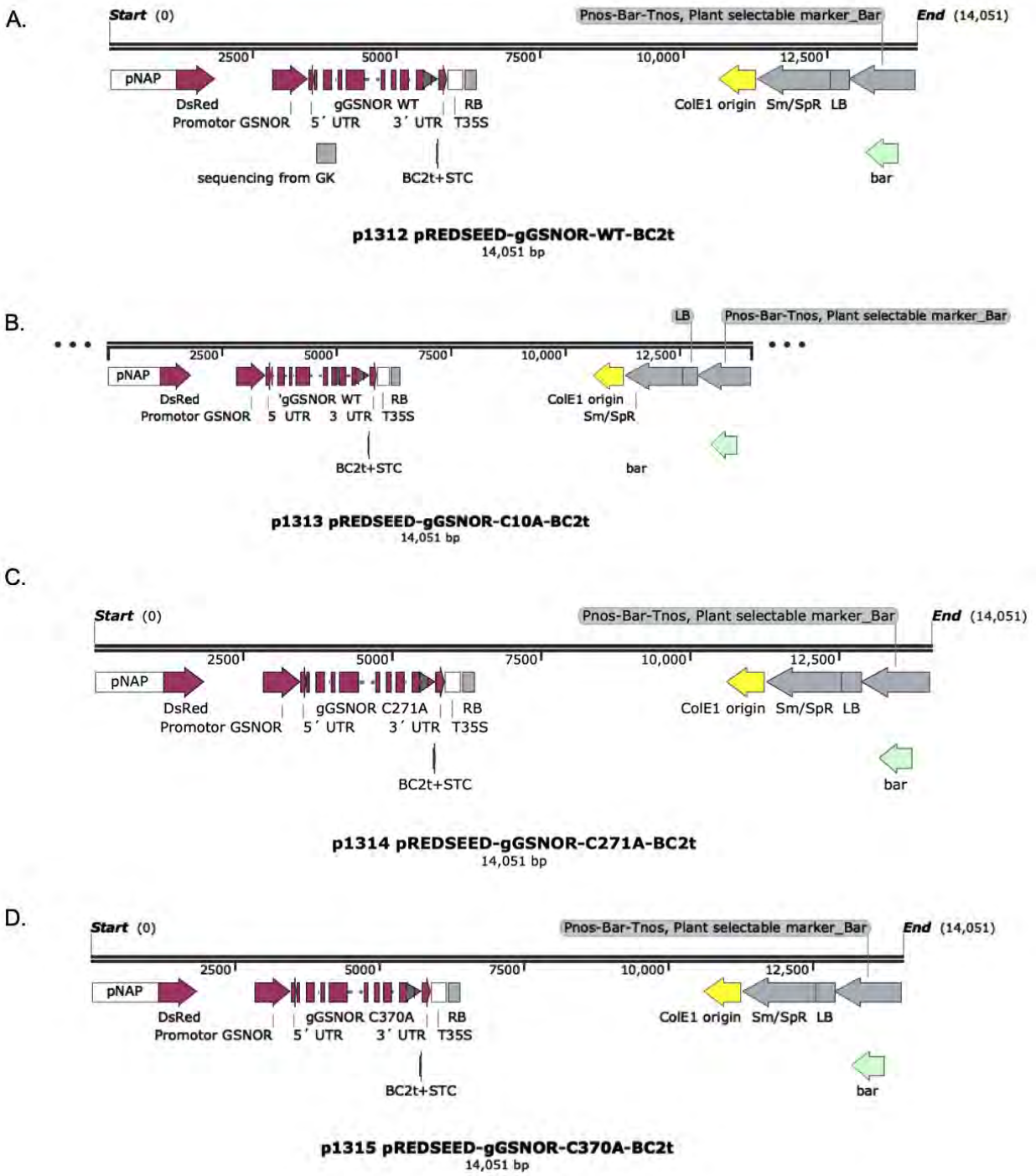

Figure 6. Plasmid constructs for transformation of transgenic lines. Plasmids used to create random T-DNA insertions in the background of the *hot5-2* mutant. Each plasmid contains a 636 bp WT promoter, Basta resistance gene, DsRed, and BC2 tag.

A second experiment was performed using the UAZ179 anti-GSNOR antibody, which has higher specificity (Figure 8B). Due to time constraints, the same protein extractions were analyzsed, thus, C370A-1 again did not have a signal. In this experiment no non-specific binding was observed, as the *hot5-2* mutant showed no reactivity. WT-1 and C10A-2 lines seemed to have more intense bands, which opens the possibility of those being overexpression lines. All

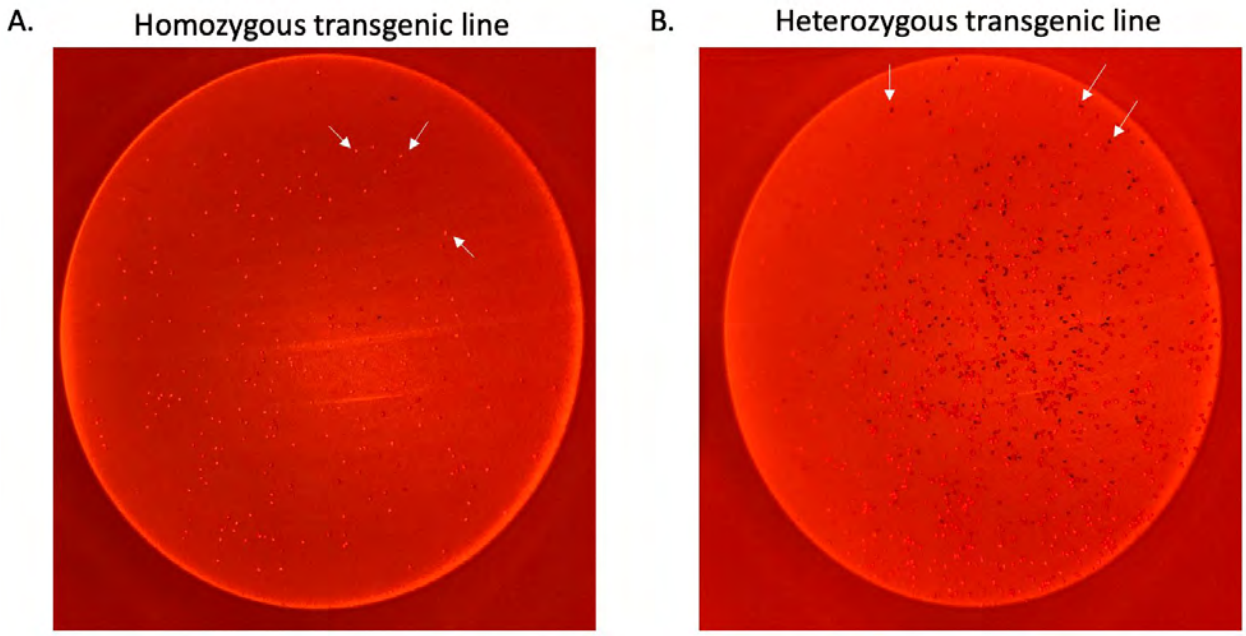

Figure 7. REDSEED Phenotype Screening of transgenic lines.

(A) Homozygous transgenic line where all seeds are glowing. The black spots observed are impurities obtained while harvesting the seedlings. White arrows indicate three of the fluorescent seeds on a plate where all seeds are fluorescent.

(B) Heterozygous transgenic line where the segregation can be observed. Some seedlings are fluorescent whereas some are not. White arrows indicate non-transgenic seeds.

transgenic lines produced bands for GSNOR protein, which further confirmed the expression of the GSNOR protein in all transgenic lines.

3.4 Growth and development under non-stress conditions

The overall growth of all transgenic lines was observed using a GoPro camera. The data

were collected for 45 days and a time lapse video was created. There were duplicates of each line and yet there was significant biological variability in plant growth. Figure 9 shows photos captured on Day 15, 25, 35, and 45 of growth. Compared to the WT and *hot5-2* controls, the overall growth of all transgenic lines was more similar to WT than *hot5-2.* At least one independent line in each construct had varying results, thus, the observation would require more replicates of the same independent lines to form a strong conclusion. The *hot5-2* control in one of the sets did not germinate, further proving the need to repeat the experiment. The GoPro video is submitted with the thesis as a separate file.

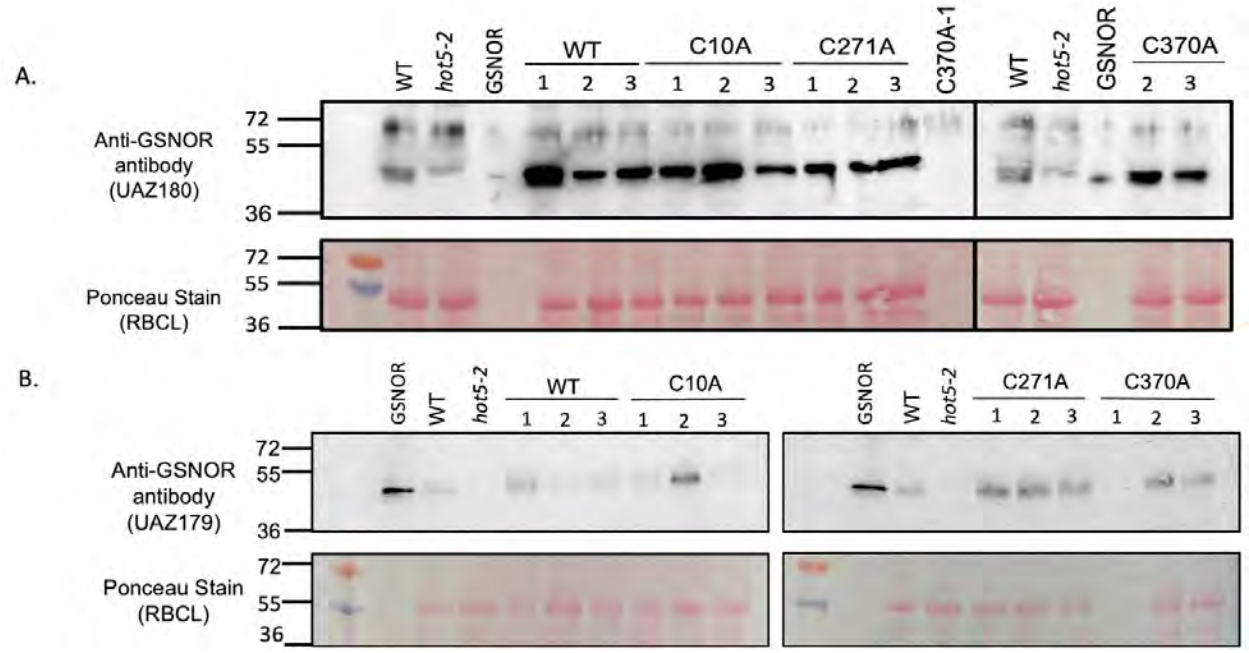

Figure 8. Western blot analysis of transgenic lines. Western blot performed using GSNOR antibodies UAZ180 (A) and UAZ179 (B) with Ponceau Stain as loading control. The numbers of transgenic lines correspond to the independent lines in Table 1. The ladder is marked on the left showing the size of the proteins in kDa. GSNOR protein is detected between the 36kDa and 55kDa markers.

The next observation was of primary root growth. Seedlings from each transgenic line were grown on MS Agar media plates and the primary root lengths were recorded after 4, 7 or 10 days of growth (D4, D7, and D10). The *hot5-2* seedlings used in this experiment were freshly

harvested. which may have affected the germination. However, compared to overall *hot5-2* primary root growth, all transgenic lines had primary root growth similar to WT (Figure 10A).

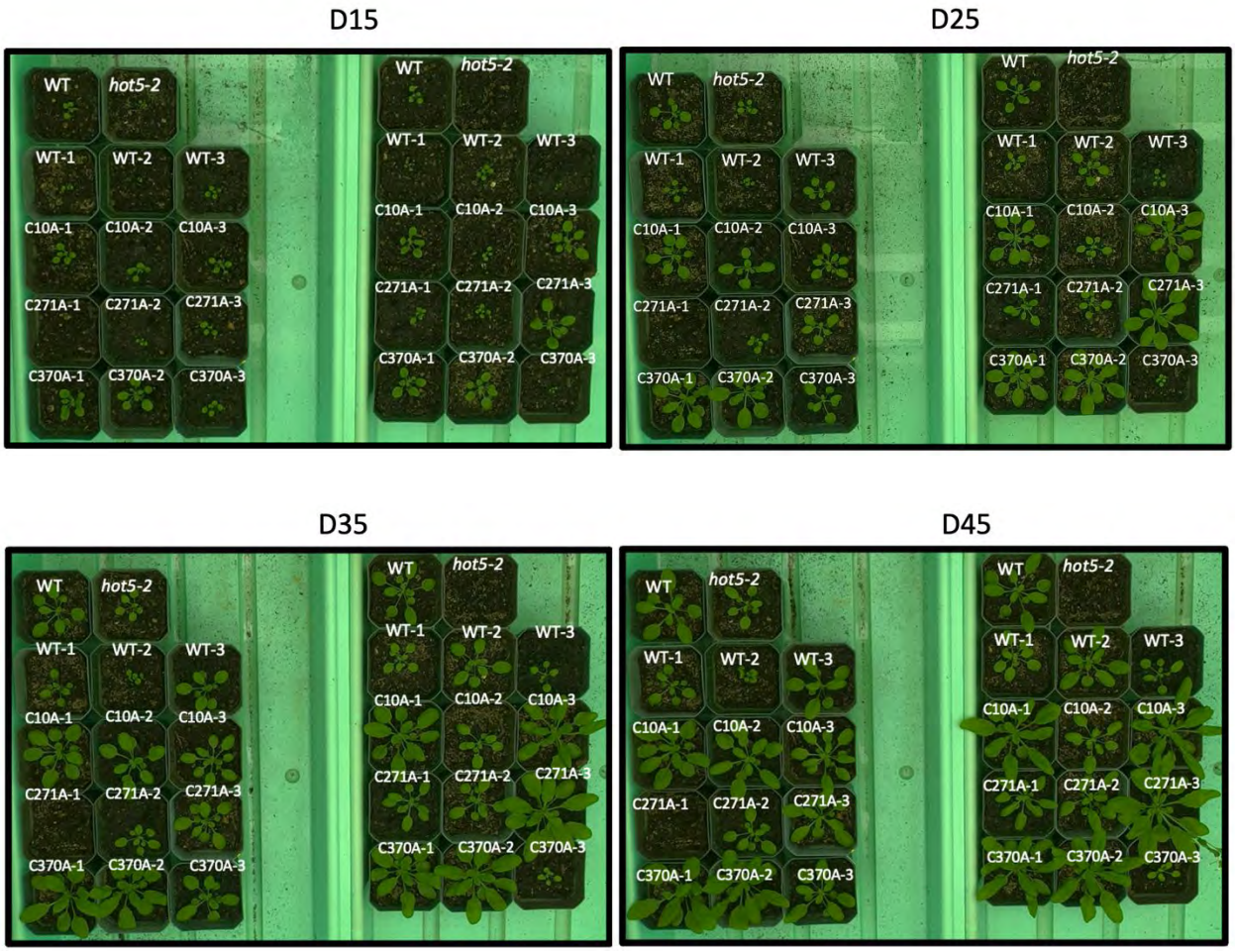

Figure 9. Growth of transgenic lines observed using GoPro Hero7 Camera. Both trays have replicates of each line. The lines correspond to the names given to independent lines in Table 1. Plants were grown for 45 days at 22°C. Figure shows photos captured on Day 15, 25, 35, and 45.

The three confirmed independent lines of C10A and two independent lines of C370A had statistically significantly higher average primary root lengths than the WT control and WT transgenic.

Next, lateral root growth was observed. *hot5-2* mutants lack the ability to grow lateral roots suggesting that GSNOR is involved in lateral root development of *A. thaliana*. All of the

transgenic lines were able to produce lateral roots, reinforcing the similarity to WT (Figure 10B). Lateral root number was higher in C10A-1 than any other control or transgenic lines. Some primary root lengths were longer than others, which may have contributed to the range of lateral root numbers. Thus, to normalize the values, the lateral root density was calculated based on lateral root number per primary root length in centimeters (Figure 10C). There was no significant difference between WT and all transgenic lines, whereas *hot5-2* had no lateral roots.

To determine the degree to which the transgenes could restore fertility to the *hot5* mutant, silique length was measured in all transgenic lines (Figure 10D). The length of the silique is directly proportional to the number of seeds per silique. *hot5-2* plants produce numerous siliques potentially a response to compensate for the low fertility phenotype. However, the size of the siliques is drastically smaller, and they also produce fewer seeds per silique, reflected in their reduced length. All transgenic lines were found to have silique lengths similar to those of the WT control and there is no significant difference between them. *hot5-2* silique lengths were significantly shorter than WT and transgenic lines, further highlighting the fertility defect caused due to the absence of GSNOR.

Collectively, all of the growth and development assays suggest replacing a cysteine residue with alanine does not hinder primary root growth and lateral root growth as the growth was similar to WT.

Lastly, trichome branching was observed in WT, *hot5-2*, and the transgenic lines. *hot5-2* plants have trichomes with two branches on their leaves, and few trichomes with three branches, and no trichomes with four branches. In contrast, WT has trichomes with three or four branches, with the majority having three, and no trichomes with two branches. Based on the results, the

transgenic lines were similar to WT, considering that all leaves had the highest composition of three branches and some trichomes with four branches (Figure 10E). Surprisingly, there was at least one trichome with two branches in each of the transgenic lines including the WT transgenic line, but no trichomes with two branches were observed in the WT control.

3.5 Heat Stress Phenotypes

Because *hot5-2* plants are heat sensitive, heat stress tests were performed to determine the heat tolerance of the transgenic lines. The first heat stress trial gave ambiguous results as the *hot5-2* samples survived the stress (Figure 11A) even though published data have documented the mutant's lack of heat tolerance (Lee et al., 2008). The *hot5-2* plants used to obtain the leaf discs for the assay were also grown from freshly harvested seeds, and the overall growth of that those plants was slower than previously observed with other batches of *hot5-2* seeds. This experiment lacked the heat sensitive control, *hot1-3,* which is a null mutant for the heat shock protein Hsp101, due to time restrictions. The appearance of each disc of the transgenic lines was also not similar. It was also difficult to visualize potential loss of chlorophyll as anthocyanin pigment accumulated in some of the leaf discs. High-light conditions or varying temperatures trigger the accumulation of anthocyanin in *A. thaliana*, as it serves as a means to protect against the severe effects of excessive light or stress in plant tissues (Das et al., 2011). A possible cause for anthocyanin could be the temperature difference as the leaf discs were obtained for plants growing at 22^{o}C, and the plates were allowed to recover at 19^{o}C after the heat stress. The anthocyanin production could have been a response to stress conditions.

In the second trial constant temperature was used for growth before and recovery after the heat treatment. Anthocyanin was reduced and the loss of chlorophyll was more clearly visible

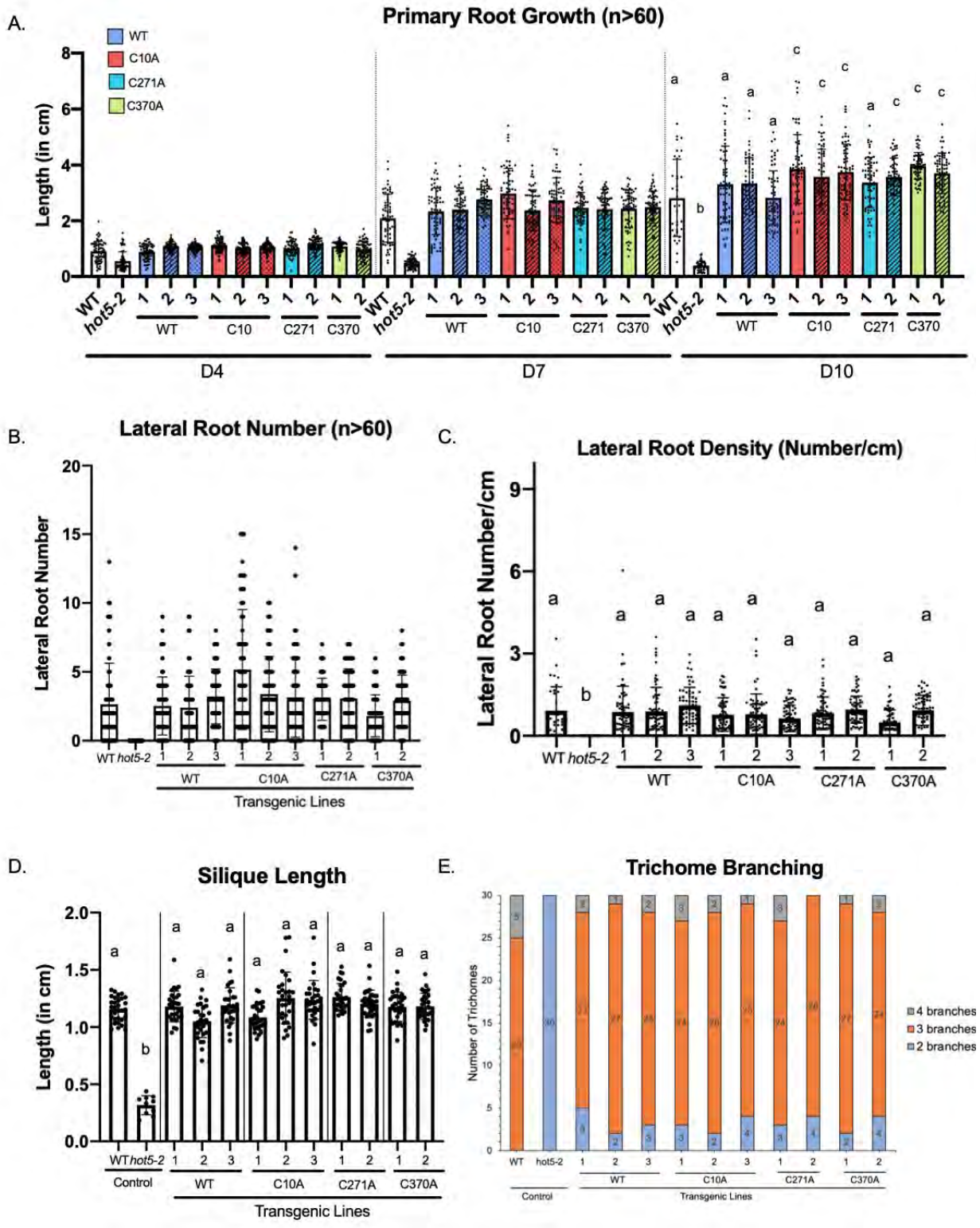

Figure 10. Phenotypic assays for WT, *hot5-2*, and transgenic lines.

(A) Data collected of n>60 seedlings and measurements of the primary root on D4, D7, and D10 of all seedlings grown on 0.5 x MS Agar media plates. Length was measured by scanning the plates and using the ImageJ software to analyze the results. Statistical

analysis performed using a Two-way Anova test. Different letter represent significant differences. Blue bars represent the WT construct, red bars represent C10A constructs, teal color represents C271A constructs, and green bars represent C371A constructs.
(B) Number of lateral roots produced by 10-day-old seedlings used in part (A). Each dot represents an individual value. A 5% standard deviation is depicted in the graphs.
(C) Lateral root density was calculated for each seedling by counting the number of lateral roots in (B) and dividing each value with the corresponding primary root length collected in (A). Statistical analysis was performed using a Two-way Anova test. Different letters represent a significant difference in values.
(D) Silique lengths measured in n=30 siliques obtained from the 5th-10th true leaf. Length recorded by taking a photo of siliques and using a ruler for measurement scale, and measuring each silique length using ImageJ. Each different letter represents a significant difference in values.
(E) Trichome branching recorded as composition of 2, 3, or 4 branches trichomes in n=30 trichomes from 2 leaves per line. Blue represents two branches, orange represents three branches, and gray represents four branches.

in most leaf discs (Figure 11B). The lack of heat acclimation was slightly more severe in *hot5-2* leaf discs. Interestingly, the C370A line seems to be sensitive to heat stress as most of the discs lost chlorophyll after heat stress. All other transgenic lines resembled the WT tolerance for heat stress.

3.6 Salt Stress

Higher accumulation of NO in plants is proposed to increase salt tolerance in *A. thaliana*, suggesting that *hot5-2* seedlings would have better stress tolerance than WT. To compare the salt tolerance of WT and *hot5-2*, salt stress experiments were performed where WT and *hot5-2* seedlings were grown on plates and transferred to 24-well plates with varying NaCl concentrations. Results showed that higher concentration of salt affects root length of both WT and *hot5-2,* as all root lengths are reduced in the 100mM concentrations and some seedlings turned yellow as well (Figure 12C). The relative root length was graphed by setting the control

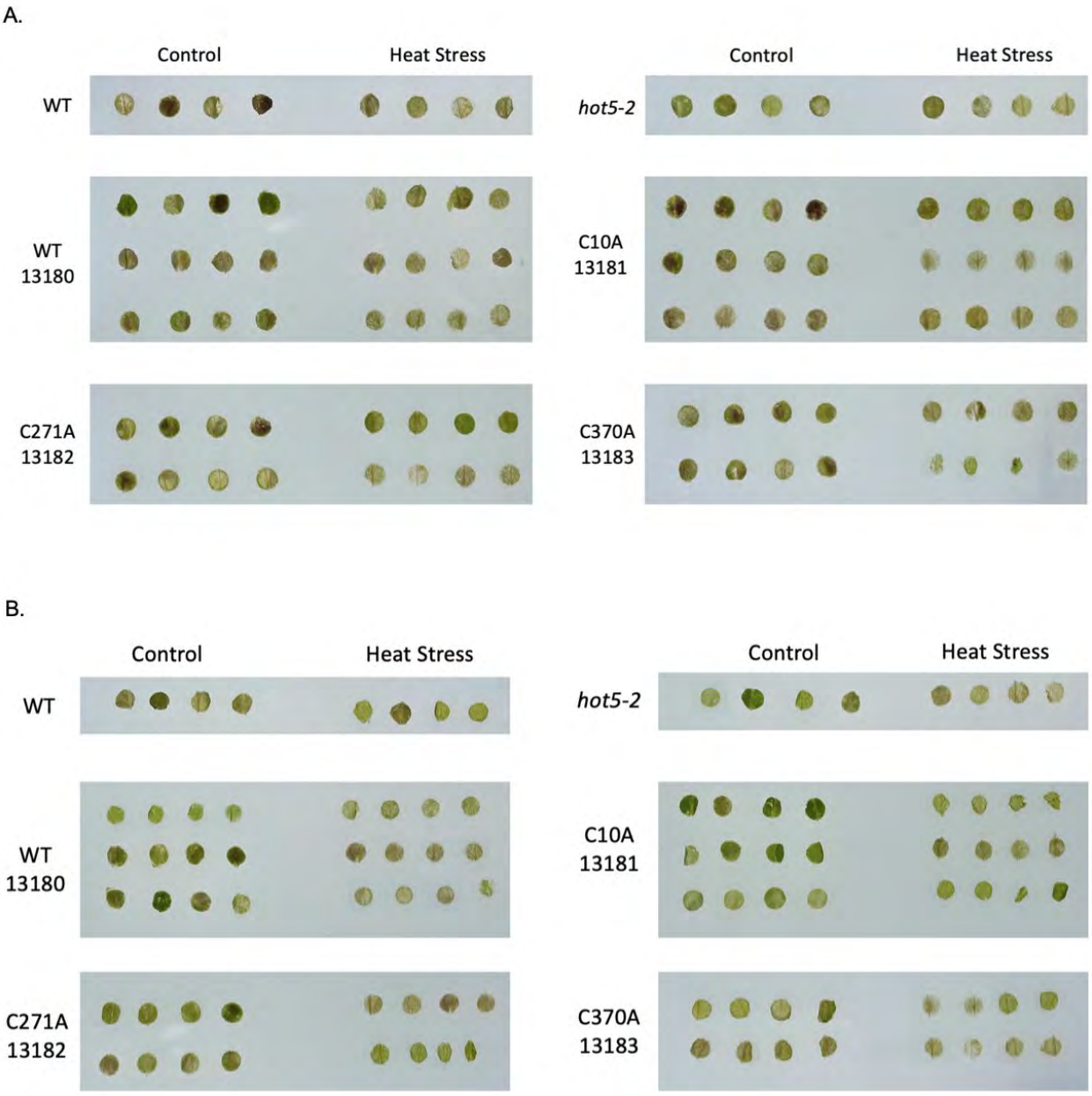

Figure 11. Heat stress assays to test thermotolerance of transgenic lines.

(A) 5mm leaf discs (4 from each lines) were floated on 2mL of MES-KOH buffer. Control was placed at 19°C. Heat stress samples were placed in 38°C for 1.5 hours, 19°C heat acclimation for 2 hours, and 48°C for 2.5. All samples were placed in growth chamber at 19°C and were photographed 5 days later

(B) Same treatment as (A), but samples were placed at 22°C after heat stress and photographs were taken 5 days later.

values to 100% and growth on the different concentrations of salt as a percentage of the control (Figure 12B). It can be concluded that salt affects root length negatively for both genotypes, and there was no significant advantage for the *hot5-2* mutant plants (Figure 12A). As the root growth of *hot5-2* seedlings is significantly slower than WT seedlings, it is possible that developmental differences cause differences in the response to salt.

For the next trial, the *hot5-2* seedlings were allowed to grow for 10 days and WT seedlings were grown for 4 days in an attempt to have roots more similar in length at the start of the salt stress treatment. Despite the changes, there was no significant difference in root length of *hot5-2* and the results were the same (Figure 12D, 12F). The relative root length graph also had similar results to the previous trial (Figure 12E). Higher salt concentration negatively affected both WT and *hot5-2* seedlings, and *hot5-2* did not have higher salt tolerance than WT seedlings.

4. Discussion and Future Directions

NO homeostasis in plants is crucial to plant growth and development, including primary root growth, lateral root development, germination rate, fertility, stress acclimation, stomatal closure, and trichome branching. NO is a reactive nitrogen species that is present in cells primarily as an adduct to GSH in the form of GSNO. The enzyme GSNOR catalyzes the reduction of GSNO to modulate the levels of NO in cells (Figure 1). GSNOR undergoes various post-translational modifications, one of which includes S-nitrosation of solvent-accessible and non-zinc chelating cysteine residues Cys-10, Cys-271, and Cys-370. S-nitrosation of these residues alters the function of GSNOR and replacing any of those residues with alanine eliminates the site of nitrosation (Figure 2).

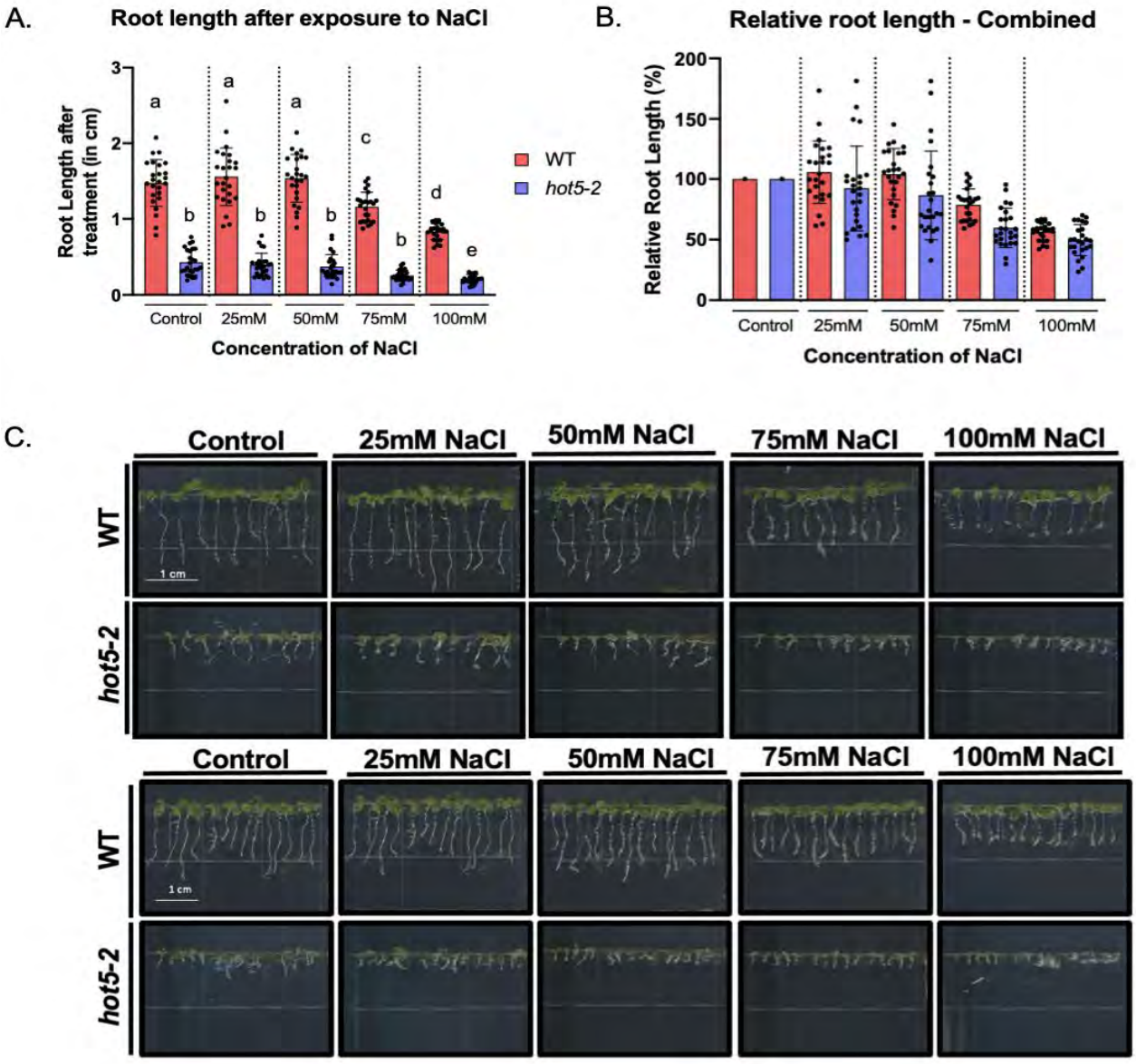

Figure 12. Salt stress assays to test salt tolerance at varying NaCl concentrations.

(A) Graph of root length (in cm) of 8-day-old seedlings photographed after 4-day exposure to 0mM, 25mM, 50mM, 75 mM, and 100mM NaCl. Red bars represent WT seedlings, blue bars represent *hot5-2* seedlings. Statistical analysis using Tukey's test ($P<0.05$). Different letters indicate significant differences.

(B) Relative root length ratio created to compare root lengths in salt concentrations to control.

(C) Scan of n=25 seedlings of 8-day-old WT and *hot5-2* grown on 0.5 x MS Agar media plates.

(D-E) Same as (A-B)

(F) Scan of n=15 seedlings of 8-day-old WT and 12-day-old *hot5-2* grown on 0.5 x MS Agar media plates

In *A. thaliana* the null mutant for GSNOR is *hot5-2,* which shows hindered primary root growth, lack of lateral root development, a multi-branching phenotype, two-branched trichomes, reduced fertility, and shorter silique length compared to WT (Figure 3). Agrobacterium mediated transformation was performed in *hot5-2* plants where C10, C271, or C370 residues were replaced with alanine to form C10A, C271A, and C370A transgenic plants (Table 1). The WT GSNOR gene was also reintroduced into *hot5-2* with the expectation it would restore the phenotype to WT. All the transgenes were driven by the WT GSNOR promoter, included a C-terminal BC2 tag on the GSNOR protein, and plants contained a Basta resistance and a DSRed gene (Figure 6) for screening.

After screening for the REDSEED phenotype, homozygous lines were isolated to be used for all assays (Figure 7). With three confirmed independent lines for the WT and C10A construct and two independent lines for C271A and C370, Western blot analysis was performed. Three independent lines were used to have conclusive results to eliminate the possibility of variation caused due the random T-DNA insertion in the transgenic lines. A possible third independent line for C271A and C370A was also used for this experiment. This was done to quantify the protein produced in each of the transgenic lines (Figure 8). *hot5-2* does not produce any GSNOR protein, and thus, no reactivity to the GSNOR antibody was observed. The first trial using the anti-GSNOR antibody UAZ180, two bands reacted in WT and all transgenic lines, and only one band in *hot5-2*. This suggested that there is another common protein being detected by the antibody in WT and *hot5-2* lines which is not GSNOR. All transgenic lines showed bands for GSNOR between 36kDa and 55kDa meaning that protein was being produced, suggesting that creating a mutation in the cysteine residue did not affect the expression or stability of the protein. The bands appeared stronger in all the transgenic lines with this antibody, which suggested the

transgenes might be slightly over-expressed, even though the constructs used the native promoter. It is possible that there was an interference in band width due to the non-specific binding of antibody, thus, the western blot was repeated.

For the second Western analysis, a more specific anti-GSNOR antibody, UAZ179, was used and the second, non-specific band, was not detected. The results were consistent with the first blot, and all transgenic lines showed slightly higher levels of protein expression than the WT control. However, C370A-1line, which is a confirmed homozygote, had an error during protein extraction, and thus this experiment needs to be repeated with new extraction from samples.

To observe overall growth of the transgenic lines, a GoPro camera was used to create a time lapse video during the timespan of 45 days (Figure 9). There was variation in the replicates of each independent line, which means that the experiment has to be repeated with more replicates to clarify potential differences in growth. However, the growth of all transgenic lines was more similar to WT control than to the *hot5-2* control.

Further phenotypic assays included primary root growth, lateral root growth, silique length, and trichome branching. Primary root growth of all transgenic lines was similar to the root lengths of the WT seedlings (Figure 10A). However, the *hot5-2* seedlings were used from a freshly harvested batch, which may have delayed the germination rate. Despite this, the transgenic lines had primary roots longer than the length of usual *hot5-2* seedlings, thus concluding that the Cys to Ala mutants are able to complement the absence of GSNOR in the *hot5-2* mutants.

Next, lateral roots were observed, as *hot5-2* lacks the ability to grow lateral roots (Kwon et al., 2012). In contrast to *hot5-2,* all transgenic lines were able to grow lateral roots, which

further showed the similarity to WT (Figure 10B). Lateral root density for all of the transgenic lines was statistically not different to WT (Figure 10C).

hot5-2 siliques are shorter and produce fewer seeds. Since the length of the silique directly correlates to the number of seeds, the lengths of the siliques were measured. The siliques of the transgenic lines resembled the average size of WT siliques, suggesting that all transgenic lines do not have reduced fertility like *hot5-2* (Figure 10D).

Lastly, trichomes in *hot5-2* have two branches, and no trichomes with three or four branches, and WT trichomes have only three or four branches. The trichomes of transgenic lines consisted of three branches as the majority, which is similar to the WT trichomes, and few trichomes had two and four branches (10E). Overall, all these phenotypic assays concluded that the transgenic lines have phenotypes similar to WT instead of *hot5-2,* meaning that the replacing the Cysteine residues with Alanine complements that WT phenotype.

For stress acclimation, heat tolerance and salt tolerance were tested in all lines. *hot5-2* mutants are heat sensitive and they lose chlorophyll after heat stress, suggesting that the mutant has lower tolerance to heat stress compared to WT (Figure 4). In contrast, *hot5-2* seedlings are expected to have higher tolerance to salt stress as others have reported that accumulation of NO provides better acclimation to salt (Figure 5). In the heat stress assay, results varied drastically. The *hot5-2* mutant seemed to have some tolerance and did not show the severe effects that were observed by Lee et al. (2008). The null mutant of Heat-Shock Protein101 (Hsp101; *hot1-3*), which has an established heat- sensitive phenotype was not included in this experiment due to time constraints. Due to the variation in results and absence of the *hot1-3* control, this experiment would have to be repeated to get affirmative results.

In the salt stress experiment, *hot5-2* did not have any added advantage to tolerance of salt, although higher NO levels, which are present in *hot5-2*, have been reported to enhance salt tolerance (Zhou et al., 2016). With increased concentration of salt, the root lengths were shorter in both WT and *hot5-2* plants, and most seedlings started to lose chlorophyll at 100mM NaCl. These results were drastically different from the results published by Zhou et al., (2016). However, a recent study showed results that were consistent with the data collected here, where it was concluded that 100 mM NaCl results in a mild stress to generate enough ROS to create the oxidative environment required to produce NO. Due to the reduced NO levels, germination was equally impaired in all the lines (Lechon et al., 2020). More trials of this experiment need to be done with mutant lines that have higher and lower accumulation of NO to compare with the transgenic lines.

Overall, the Cys to Ala mutations of the C10, C271, and C370 residues were able to complement the phenotypes of WT plants. S-nitrosation was shown to inhibit GSNOR activity in vitro (Guerra et al., 2016), and the higher number of NO moieties on the GSNOR dimer decreases activity. When one nitrosated residue is absent, the transgenic lines are able to have normal plant growth which is similar to the WT control plants.

A recent publication by Zhan et al. (2018) concluded that NO is involved in autophagic degradation of GSNOR by S-nitrosation of the C-10 residue. They observed that nitrosation of the C-10 residue induces conformational changes that facilitate a selective autophagy response in hypoxia conditions. Similar root-stress experiments could be conducted to determine if C-271 and C-370 residues are also crucial in stress responses of *A. thaliana*. My work has established transgenic lines and assay conditions that can be used in additional experiments to investigate further the effects of these cysteine to alanine mutations in GSNOR. Further research needs to be

conducted to define the roles of each of the cysteine residue in *A. thaliana* and determine how s-nitrosation of each residue affects the plants.

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