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Investigation of the Role of Overexpression of PsbS Under Stress Inducible and Constitutive Promoters to Improve Water Use Efficiency.

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Abstract

As climate change continues to impact environmental growth conditions, it has become increasingly more important to identify potential mechanisms of crop development to resist these changes. Previous studies have identified the role of PsbS in the non-photochemical quenching (NPQ) mechanism in the plant by identifying its direct effect on the rate in which excitation energy absorbed by photosystem II is dissipated as heat. It was identified also that PsbS via NPQ oxidizes chloroplastic quinone A (Q_A) which is a signal for stomatal opening in response to light. By identifying this relationship between PsbS and the signal for stomatal opening in response to light it is hypothesized that the gene has a potential vitality in the plants water use efficiency. This study aims to identify PsbS impact on the photoprotection system as well as water use efficiency through overexpression of the gene under stress inducible promoters. Our results show that overexpression of PsbS did result in increased water use efficiency in pre flowering transgenic tobacco.

Key words: Biochemistry, PsbS overexpression, NPQ, drought inducement, qE, water use efficiency

Chapter 1. Introduction

The photosystem II subunit S of protein (PsbS) is a pigment binding protein that is present in all plants including, *Arabidopsis thaliana*. PsbS is a part of the light harvesting complex family but unlike other proteins present in this family, it has four transmembrane helices. This allows PsbS to be activated by the pH changes that occur in the thylakoid lumen. In addition, the PsbS protein has a unique folding pattern that occurs when under lower pH conditions, while maintaining the dimeric form of the protein (1). Due to the unique folding of the protein, PsbS is unable to bind

to chlorophyll and carotenoids in the same way as other light harvesting complexes (LHCs). The studied structure of PsbS in addition to previous work utilizing T-DNA knockouts, indicate that the protein has a role in the non-photochemical quenching (NPQ) through its direct effect of the rate in which excitation energy is released as heat.

Non photochemical quenching (NPQ) is the process by which a plant quenches excited chlorophylls to dissipate the excess excitation energy as heat and in turn, protect the plant against photo-oxidative damage. NPQ mechanism within a plant can be induced or reduced in response to differences or changes in light intensity. NPQ can be divided into three different components based off the relaxation kinetics in darkness and the response to different inhibitors (2). The most rapid form of NPQ, qE, is an energy dependent mechanism within plants that responds well to fluctuating light intensity (3). A decrease in pH within the thylakoid lumen is a signal based on the excessive light that triggers the feedback regulation of light harvesting of qE (2). This mechanism, qE, requires PsbS, low lumen pH and de'epoxidized xanthophylls, zeaxanthin and antheraxanthin (2).

Additionally, it has been found that PsbS may act as a pH sensitive initiator of the qE mechanism when the plant is under high light conditions, or lower pH conditions (1). Through various studies, it has been shown that the level of PsbS limits the qE capacity within plants and therefore, the photoprotection system in the plant (1). Knowing that the PsbS protein is involved in the NPQ , specifically with its relationship with chloroplastic quinone A, a factor involved in stomatal signaling, it is hypothesized that an overexpression of the PsbS gene under stress inducible and constitutive promoters will result in an improvement of water use efficiency in the plant. Increased levels of qE can be attributed to the prevention of over-reduction of photosystem II electron acceptors and therefore, will lead to a larger resistance to environmental stress (5). An

overexpression of PsbS will also allow for less stomatal opening by affecting Q_A under high light stress and inevitably decrease the amount of water loss through the stomata, improving the overall water use efficiency of the plant (2). In total, an overexpression of PsbS will lead to more efficient water usage and photoprotection within various crops.

Constitutive and inducible promoters are utilized differently based on their varying advantages in overexpression of genes. Constitutive promoters are always activated and do not rely on specific environmental clues. In contrast, inducible promoters rely on certain environmental clues like e.g., abiotic stressors. Plant growth and productivity are highly impacted by water stress. The water use efficiency of a plant is a selection trait since plants have come to adapt to adverse environmental conditions or water stress. There are various molecular networks that are involved in the stress response of plants including, regulatory events mediated by abscisic acid (ABA) signaling, ion transport, and the activities of transcription factors (TFs). ABA is rapidly produced during a drought which triggers stomatal closure and stimulates a signal pathway that causes and increase in the production of reactive oxygen species. Therefore, with a stress-inducible promoter, the expression of the PsbS may be linked to the various responses in a plant that occur due to environmental stress.

Chapter 2. Materials and Methods

2.1 Identifying constitutive and drought-inducible promoters

Four stress inducible promoters were identified as driving the overexertion of genes during drought. In other words, these promoters were activated via response of the plant to drought. Responses like gene expression and signal transduction that are prevalent amongst transcription factors such as the ABA Responsive Element Binding Protein (ABRE) are found to be important

for drought resistance within plants. This protein is involved in signal transduction by binding to the ABA response element found in the promoter region of the ABA- inducible genes. The promoter RD29A was identified as having significance to drought resistance due to the fact that it has several ABREs. In Arabidopsis the promoter combines with the transcription factor, DREB1A, that specifically interacts with the dehydration responsive element (3). The second promotor chosen was ELIP2. ELIP2 is a high-light-induced protein that belongs to the chlorophyl a/b binding subfamily. Expression of ELIP2 is initiated by stresses related to photoinhibition and cold temperatures (11). The third promotor, RAB17 was chosen due to its induction by ABA (12). The final promotor, WRKY33 is a drought induced WRKY gene that is linked to stress induced processes via ABA (13). The transcription factor coded by WRKY33 had already been identified to promote root growth and show lower rate of water loss under stress inducible conditions.

2.2 Designing constructs

Plasmid map construction was done through identification of various essential components and the connection of these components into one construct using the plasmid Editor software ApE. Resulting T-DNA sequences into a plasmid background included designated direction, backbone, promoter, 5'UTR sequence, coding (SC) sequence, and terminator for two expression cassettes per construct. The selectable marker in plants was the same for each contract while the expression cassette for the gene of interest was unique in each construct in the sense that four different drought/stress inducible promoters were used. Both L1, level 1 fragments, and L2 level 2 fragments were constructed. Level 1 fragments are used to determine the position and orientation of the gene in the final construct, and they contain a different restriction site and resistance antibiotic than level 2. Level 2 constructs have two inverted Bpil sites, specific

enzyme cut sites that surround the target gene, from the insertion of the levels 1 and they allow for insertion of the gene into the vector. The following L1 constructs were created after subsequent design in ApE software: pL1M-R1-2x35S::BAR::35S, pL1N-F2-Atrd29A::AtPsbS::NOS, pL1N-F2-AtWRKY33::AtPsbS::NOS, pL1N-F2-AtAab18::AtPsbS::NOS, pL1N-F2-Atrd29A::AtPsbS::AttHSP18.2, pL1N-F2-AtWRKY33::AtPsbS::AttHSP18.2, pL1N-F2-AtAab18::AtPsbS::AttHSP18.2. The finished L2 constructs were designated as pUNL14, pUNL15, pUNL16, and pUNL17 in order as described above.

2.3 Making constructs via Golden Gate reaction

Golden gate assembly protocol was taken from Engler et al. 2009. 1 µl of miniprep DNA was diluted in 100 µl of water. 100 ng of vector backbone and 100 ng of each assembly piece was added to a total of 15 µl assembly reaction: 1 µl vector backbone, 1.5 µl NEB T4 ligase 2 million cohesive units; 1.5 µl 100x BSA, 1 µl 10x NEB T4 buffer, 2 µl Bsa1 was added for level 1 and 2 µl Bpil for level 2,. The assembly reaction was performed in a thermocycler using the following program 5 min 37°C, 3 min 37°C, 4 min 16°C for 40 cycles followed by10 min 16°C, 10 min 50°C, 10 min 80°C for 1 cycle.

2.4 Transformation of plasmid into competent E.coli cells

Transformation of plasmid into competent *E.coli* cells was performed with 1 µl of the assembly reaction into 20 µl competent *E.coli* and a diagnostic agarose gel was ran to check for successful assembly. LB media plates were prepared by dissolving 10g of tryptone, 5g yeast extract,and 10g NaCl in 950 ml deionized water. Next the pH of the medium was adjusted to 7.0 using 1M NaOH followed by bringing the volume up to 1 liter. The solid media was made with an addition of 15g/L of agar. The media was sterilized by autoclaving for 20 minutes at 15 psi. Before

addition of appropriated antibiotic to the media the solution was cooled to 55°C. The antibiotic used in the gels was kanamycin at 100ng/ml. Until used, the media was stored at 4°C in the dark. To begin the transformation protocol, tubes of E. coli cells were thawed on ice for 10 minutes. The tubes were flicked to mix and then two 25 µl of the E. coli were taken and placed into separate tubes and placed in the ice (label according to construct). Tubes were flicked to mix and then 1µl of plasmid DNA was added by placing a drop along the edge of the tube above the E. coli and flicked 4-5 times to mix the cells and DNA. The tubes were placed into the ice bath for 30 minutes. Next the heat shock was performed in a water bath set at 42°C for 30 seconds. After the heat shock tubes were incubated on the ice for 5 minutes after which 950µl SOC media was pipetted into the mixture and then placed into the shaker at 37°C for 60 minutes at 250 rpm. Before plating the transformation mix was diluted by a preferred dilution factor and 100µl of this mix was spread onto the LB media and incubated at 37°C overnight.

2.5 Isolation of DNA using the miniprep DNA kit

The colonies from the plates were picked up with a toothpick and placed into vials containing liquid LB media. The vials were then placed in the shaker for 16 hours at 350rpm. DNA was extracted from the plasmid using QIAprep spin miniprep kit according to the manufacture protocol with one exception of the final elution buffer being replaced with 50µl of dH2O.

2.6 Plant transformation

Plants were transformed using tobacco transformation protocol outlined in citation 4.

2.7 Polymerase Chain Reaction

Plasmids were tested for confirmation of right assembly using polymerase chain reaction (PCR). The following PCR program was used with multiple set of primers to produce initial gel electrophoresis genetic confirmation results: 95°C for 2 minutes, 95°C for 0.3 minutes, 55°C for

0.30 minutes, 72°C for 1 minute, 72°C for 5 minutes and held at 12°C after 40x cycles. DNA from minipreps was run with two sets of primers, BAR3F+R and PsbS_4F+R.

2.8 Growth of transformed plants for physiological study

The seeds of Four T0 lines were chosen for physiological study based on preliminary PCR results. The studied lines included 14.1 P2D2 (designated here as UNL14), 15B P2D9 (designated here as UNL15), 16A P7D3 (designated here as UNL16), and 17A P2D2 (designated here as UNL17), wild type (WT) , and T2 homozygous NPC line, a positive control line with constitutively overexpressed PsbS. The seeds were planted on metromix 2.0 soil in four 96 well plates with 8 plants per line resulting in 48 plants per plate. Plates were grown in Beadle Greenhouse, in a growth chamber with 8.5-hour-long day. The seedlings were sprayed with water every day after seeds planting.

Seeds from the same lines were grown until the preflowering stage for further physiological analysis.

Plants were grown in 1L pots on metromix 2.0 soil in greenhouse settings in Beadle Greenhouse. The fertilizer was given through a hose every other two days with unfertilized water every day in between. This watering schedule was changed as plants grew to being watered every other day. Both the seedlings and the preflowering plants underwent a drought-controlled treatment . The seedlings underwent drought stress three days after their germination and preflowering plants underwent drought stress after the growth of four fully expanded leaves. The NPQ was measured on 30-day old seedlings through imaging with the fluorescent imager. After the first day of control measurements, where the seedlings were given water, seedlings underwent drought stress, lack of watering, for three subsequent days and were imaged in the morning every day. The final day, the seedlings were watered in the morning and NPQ data was collected at the hour

mark and four-hour mark after watering. Pre-flowering plants were allowed to continuously grow until they reached their pre-flowering stage, about 4 fully expanded leaves of growth. They were imaged for NPQ using a fluorometer and measured for stomatal conductance using a porometer. They were also sampled using a 4-disc puncher to determine water content of the leaves.

2.9 Water Content of leaf discs

To obtain the water content of the leaf in pre-flowering, four leaf discs were hole punched out from fully expanded leaves from each plant and collected in a glass vial. The glass vials were filled with water and the discs were left to soak overnight in room temperature conditions. The discs were then removed from the vial in the morning and weighed on an analytical scale. The weights were recorded, and the discs were then wrapped in weighing paper and placed in the oven at 65°C to dry for 8 hours. The dried discs weighed. and in order to establish the water content the initial weight was subtracted from the dried weight.

Chapter 3. **Results**

3.1 Physiological Phenotype in seedling stage

After seed germination of the six lines, the seedlings were analyzed through photographic RGB and fluorescence imaging. Transgenic seedlings seemed to indicate a phenotype of larger and more abundance of leaves as well as slightly greener color. Phenotypic comparisons as well as fluorescence comparisons of a seedling in UNL14 line caring construct pL1N-F2 AtAab18::AtPsbS::NOS in comparison to the WT is shown in Figure 1.

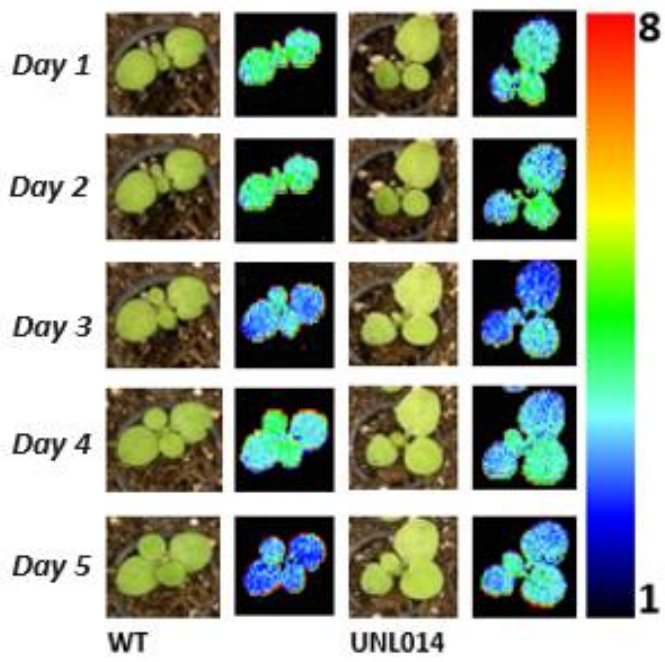

Figure 1. RGB and fluorescent pictures comparison between single WT and UNL14 seedling. NPQ bar is shown on the right indicating the color associated with the value of NPQ. Day 1 indicates control, Day 2-4 indicate drought stress, and Day 5 is recovery.

NPQ of seedlings were determined through fluorescence images. The significant differences between WT and transgenic plants are demonstrated in Figure 2. This graph shows that there are differences between the WT, positive control (NPC), and seedlings of transgenic lines in NPQ kinetics. Multiple seedling NPQ has increased in comparison to the wildtype on day 1 of control and on day 1 of recovery (5^{th} day of experiment) the seedlings were more comparable to the positive control. Figure 3 shows the average NPQ max of each line in comparison to the wildtype and positive control on each day of the experiment. The T-test analysis was in order to compare the transgenic seedlings to the wildtype. NPQ max was significantly higher in all four transgenic lines in comparison to the wildtype on day 3 and 5 of experiment. In addition UNL14

(day 2 and 4) and UNL15 (day 4) and UNL17 (day 1, 2 and 4) had also significantly higher than WT NPQ max in other days of treatment.

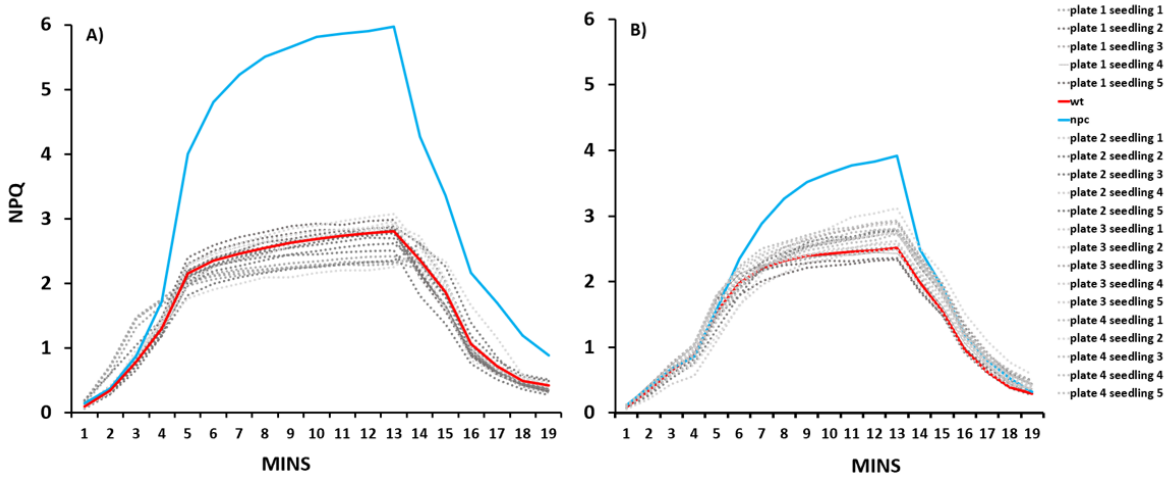

Figure 2. The NPQ kinetics induction (light; first 10 minutes) and relaxation (dark; last 10 minutes) of UNL14 seedlings in comparison to the WT and positive control, NPC12 on day 1 (left) and day 5 (right) of drought treatment.

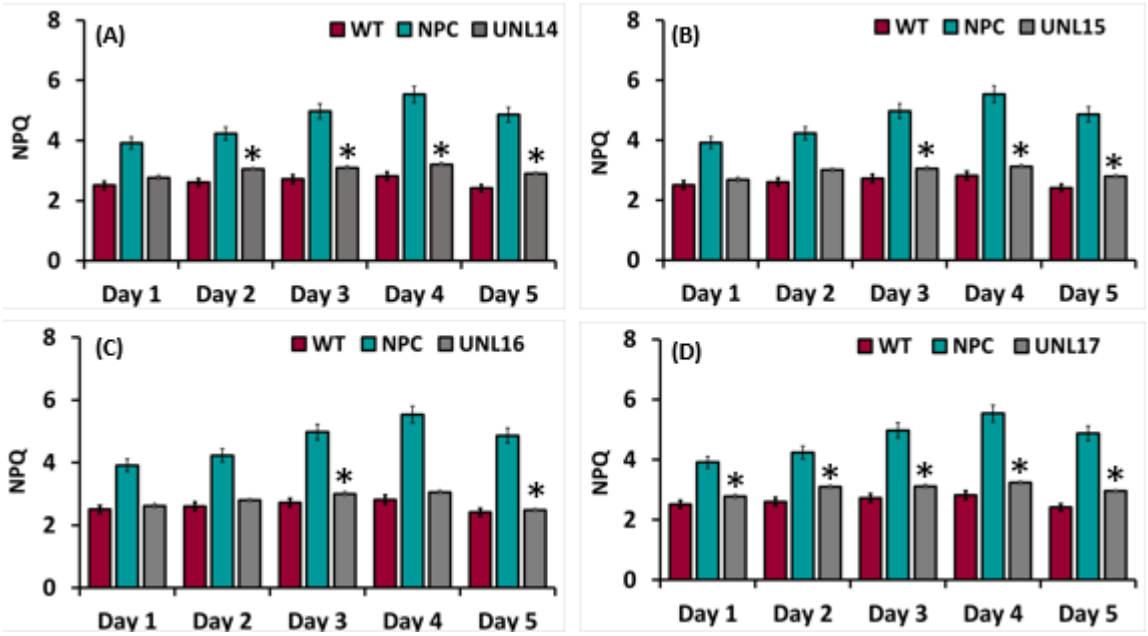

Figure 3. Average NPQ of 48 seedlings from each line in comparison to the WT and positive control NPC for each day of the experiment. Statistically significant differences ($p < 0.05$) between WT and transgenic lines are indicated with an asterisk. Error bars show standard error of the mean.

3.2 Physiological Phenotype in Pre-Flowering stage

Figure 4 shows the maximum NPQ of transgenic lines in comparison to the wildtype during each of the 5 days of the experiment where day 1 is the control, days 2 through 4 are drought conditions and day 5 is the recovery. The graph shows that the change in NPQ is higher in transgenic lines UNL16, and UNL14 on days 1 and day 5,respectively (Fig.6 and7). In addition, there is an increase in line UNL17 on the second day of drought and recovery (Fig. 4 and fig. 5).

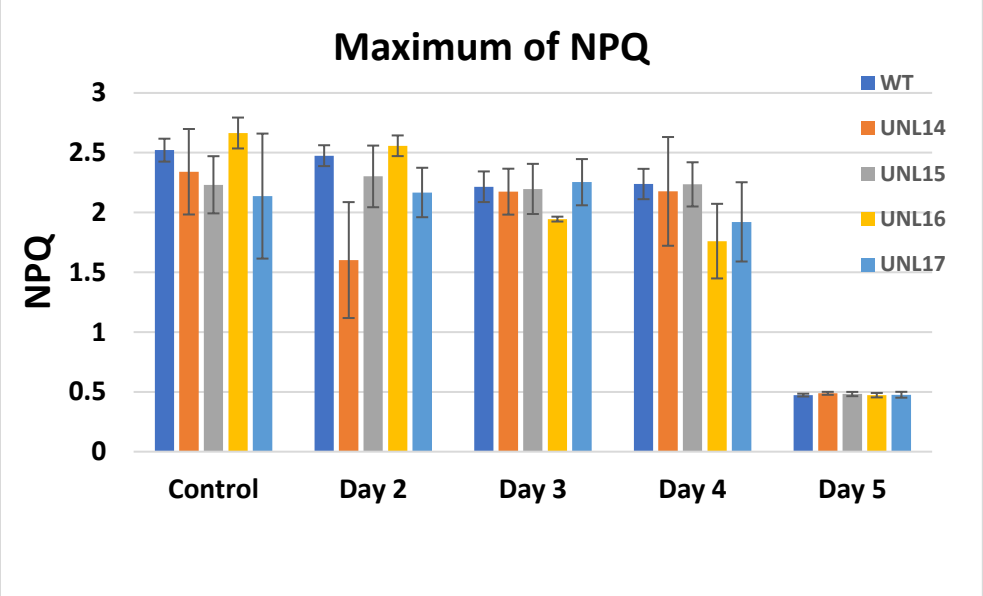

Figure 4. Average maximum NPQ of all lines in comparison to the WT. Error bars show standard error of the mean.

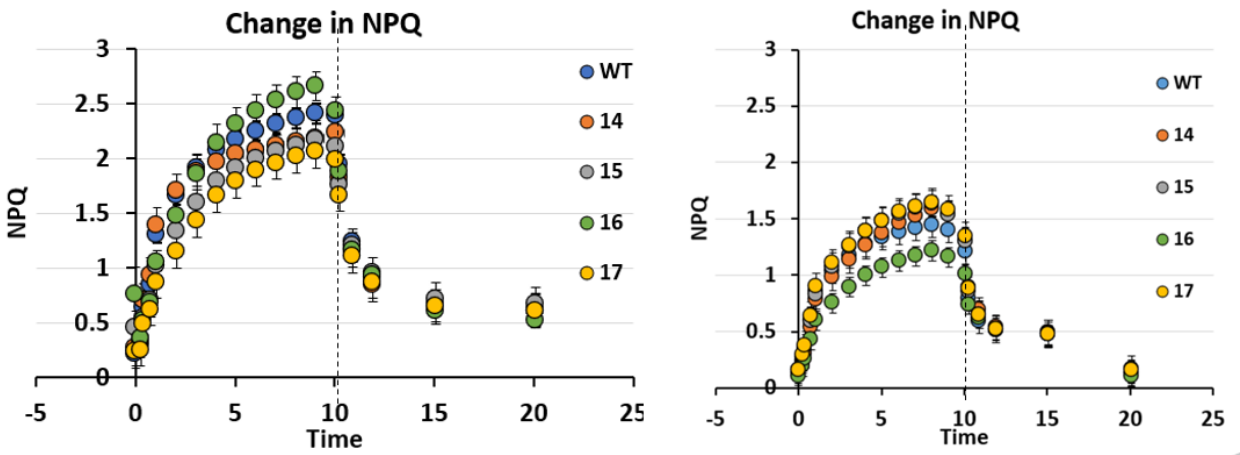

Figure 5 Changes in NPQ kinetics induction (light; first 10 minutes) and relaxation (dark; last 10 minutes) in four transgenic lines and corresponding WT on day 1 (left) and day 5 (right) of drought treatment of plants in pre-flowering stage. Error bars show standard error of the mean.

3.3 Stomatal conductance in pre-Flowering stage

Porometer was used for measurements of difference in stomatal conductance (gs) between the wildtype and transgenic plants. Noticing the trends of a slight increase in NPQ between some of the lines in comparison to the WT leads to the idea that they would have a decrease in stomatal conductance under the same conditions. Fig. 6 denotes the average stomatal conductance on T1 generations of four transgenic lines and corresponding wildtype. Based on the graph created using the data from the porometer, there doesn't seem to be a trend in a decrease or increase of stomatal conductance between the transgenic lines and the WT.

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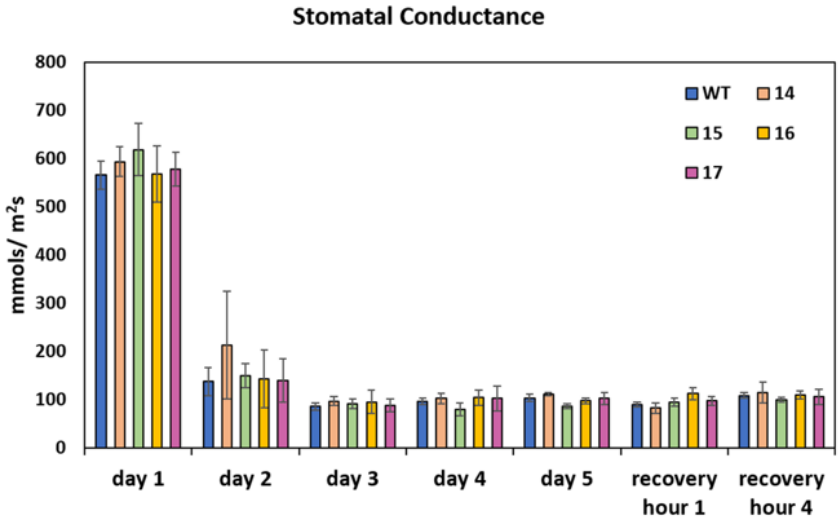

Figure 6. Stomata conductance for four transgenic lines in comparison to the WT. Error bars show standard error of the mean.

3.4 Water content in pre-Flowering stage

Water content measurements were taken during the control day and on day 4 of drought conditions. Fig. 7 shows that the transgenic plants had a smaller change in water content after drought.

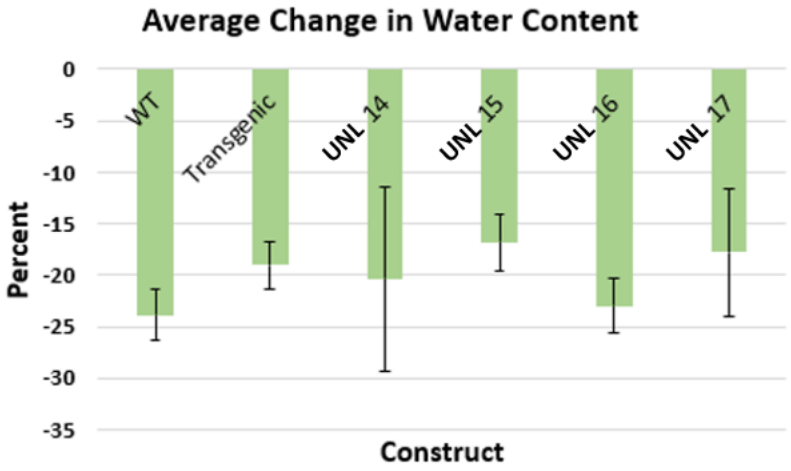

Figure 7. Water content difference between day 1 and day 4 denoted by percentages. Average measurements of each line (UNL14, UNL15, UNL16, UNL17) are graphed as well as a total average of all transgenic plants (Transgenic) in comparison to the WT. Error bars show standard error of the mean.

Chapter 4. **Discussion**

The ultimate goal of this study was to identify whether or not PsbS has an effect on the overall water use efficiency of tobacco under drought conditions. Overexpression of PsbS was tested under four drought inducible promoters and physiologically studied in order to identify whether the gene increases the overall water use efficiency as well as increase the photoprotection mechanism, NPQ. The initial hypothesis was based on the known relationship that PsbS has on the qE of NPQ, redox state of QA and the signal for stomatal opening in response to light. Knowing that PsbS protein is related to the stomatal signaling mechanism, it was hypothesized that an overexpression of the PsbS gene will increase the water use efficiency.

This study looked specifically at both the stomatal conductance and the water content of the plants in order to identify whether the plants had increased water use efficiency. The stomatal conductance data taken from the porometer didn't show any trends between the transgenic and the WT however, the water content data showed that the average water content of all transgenic lines had a decreasing trend in percentage difference in comparison to the wildtype. This is a positive result that it shows the water use efficiency of the PsbS overexpressed lines maintained higher water content than the wildtype. This also supports our primary hypothesis that an overexpression of PsbS can lead to an increased in water use efficiency. The contrasting data between the porometer data and the water content data can be attributed to the difference in the time of day in which the samples were taken, and the large error bars can be attributed to the lower number of biological replicates between the samples or the fact that the plants sampled were the T1 heterozygous generation. Future research with the T2 homozygous generation can decrease the difference between the biological replicates and therefore, decrease the standard error of the means what would help in finding the significant difference between studied lines.

Previous research has already concluded that PsbS has a role in NPQ and that it is mostly associated with the NPQ max (8). The NPQ max of UNL14 and UNL17 were increased on day 5 of recovery in comparison to the WT. Both the seedling and the preflowering data seemed to show an increasing trend in NPQ in transgenics in comparison to the wildtype. Although the trends seem to have an increase in NPQ on day 5 of relaxation and in increase in NPQ max for both the seedling and the pre flowering data, PCR data confirming the successful transformation of the plasmids has yet to be done. Further analysis needs to be completed in order to validate the study and identify whether or not the plants were successfully transformed via using e.g. ddPCR, RTqPCR and western blot.

PsbS is universally conserved gene and therefore, manipulation of the gene can be efficient in all crops. Its overexpression has been shown to alter the NPQ of plants as well as having the potential to alter the water use efficiency through direct effect of the stomatal opening machinery in response to high light conditions. Utilizing its relationship with stomatal opening, the gene can be effective in increasing water use efficiency and combatting impending climate change. Knowing a gene that can increase water use efficiency as well as protection under high light conditions is proving to be essential when looking at potential techniques to improve crop yield and growth under drastic climate change conditions.

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