OPTIMIZING CROP CULTIVATION ON THE INTERNATIONAL SPACE STATION BY IMPROVING PLANT DESICCATION-TOLERANCE WITH TARDIGRADE PROTEINS

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Abstract

Despite advancements in crop growth on the International Space Station (ISS), unforeseen dry conditions have highlighted the demand for research into enhanced plant desiccation-tolerance. Tardigrades are extremotolerant microorganisms which can survive abiotic conditions such as drought, radiation, and extreme temperatures. This study successfully establishes a method to introduce tardigrade intrinsically disordered proteins (TDPs) in a plant vector, *Nicotiana benthamiana*, via agroinfiltration. Five TDP constructs were designed and tagged with mVenus, a yellow fluorescent protein to visualize TDP expression. The TDP expression constructs were successfully co-infiltrated with an abscisic acid (ABA) autoluminescent reporter in a transient transformation of *N. benthamiana* leaves via agroinfiltration. After three days, the leaves were excised from the plants and subjected to either dry conditions or floated on water. Luminescent imaging of whole leaves was used to evaluate osmotic stress and ABA-induced luminescence over time. While our initial constructs failed to express the TDP-mVenus fusion, we validated our methods for measuring luminescence reporters of ABA signaling in response to the simulated drought stress in detached leaves. In addition to agroinfiltration with corrected constructs, floral dip of *Arabidopsis thaliana* will be used to generate transgenic lines for further examination of the effects of TDP expression on plant desiccation-tolerance.

Introduction

Crop growth in space conditions is critical to long-term, independent space travel. NASA has made tremendous progress with innovative plant growth apparatuses, such as "Veggie" and the Advanced Plant Habitat (APH), to accommodate plants to the stressors of space. With "Veggie," scientists have successfully grown leafy vegetables such as lettuce, Chinese cabbage, red kale, mustard, zinnia flowers, and *Arabidopsis*. In the "Veggie" apparatus, plants grow in wicking "pillows" which help bind the roots and contain fertilizer and injected water. Wicks and "pillow" structures distribute water evenly throughout the growth medium and help plants passively absorb water and nutrients[11]. Unfortunately, a recent failed mission, VEG-05, highlights the demand for further research to optimize cultivation of other crops, like fruits, on the ISS. VEG-05, launched in 2022, aimed to grow over 100 tomato fruits in orbit. However, before germination could occur, there was a sudden drop in humidity. The decrease in moisture caused the wicks and soil to dry up. Added water to attempt rehydration resulted in death due to water pooling, suffocating the young plants. Ultimately the experiment yielded only twelve tomato fruits[3]. Even in the controlled growing conditions of the ISS, abiotic stressors found on Earth, such as drought, proved lethal to the moisture-dependent plants.

On Earth, climate change poses a dire threat to plant life and global agriculture as increasing temperatures and extreme weather increase the likelihood of drought. Without water, plants cannot undergo photosynthesis, thus hindering growth and threatening crop yield[4]. The natural responses of plants to osmotic stress, such as wilting/slowing growth rates and stomatal closure to limit water loss via decreased transpiration, are mediated by

plant hormones like abscisic acid (ABA)[10]. These natural responses are not always sufficient to protect plants, like tomatoes, that are not well-adapted to survive periods of drought.

Tardigrades, also dubbed "water bears," are renowned as extremely tolerant organisms; with the ability to survive desiccation, temperature extremes, radiation exposure, and even the vacuum of space. Like plants, when dried, tardigrades reduce their surface area and slow their biological activity, by retracting their limbs into their bodies and hardening into a "tun" state[5]. Tardigrades are anhydrobiotic organisms, meaning they can lose nearly all their body's water content (>95%). In this desiccated state, tardigrades may be able to survive over a century[5].

Microorganism desiccation resistance was primarily attributed to accumulation of bioprotectants like trehalose. However, recent studies determined that tardigrades accumulate minuscule amounts of trehalose, suggesting that other mediators work synergistically with trehalose to achieve tardigrades' robust resistance[12]. Such novel mediators are known as tardigrade intrinsically disordered proteins (TDPs), exclusive to tardigrades. There are three known TDPs: cytoplasmic-, secreted-, and mitochondrial- abundant heat soluble (CAHS, SAHS, MAHS) proteins[5]. TDPs are enriched and highly active during desiccation and are required for tardigrade survival during the drying process[2]. Recently, the roles of engineered CAHS variants, CAHS D, gelling and non-gelling, in water retention during drought conditions were analyzed[13]. Though CAHS D was determined to not physically retain water, the gelling CAHS variant interacts with water molecules and may confer improved desiccation-tolerance compared to the non-gelling variant[13]. A recent study of CAHS expression improved desiccation-tolerance nearly 100-fold in yeast, with similar results in *E.Coli*[2]. Additionally, studies which induced radiotolerance in tobacco plants and human cells, as well as hyperosmotic tolerance in human cells, via TDP expression suggest that tardigrade desiccation-tolerance can be conferred to more complex organisms, like plants[6,15]. Thus, this study aims to develop a method to express these five TDPs in plants to analyze different TDPs' effects on plant desiccation-tolerance. This research could ultimately yield transgenic seeds and optimize crop growth in orbit by preconditioning crops to be resilient to abiotic stressors.

Results and Discussion

Expression Construct Generation

To test the potential of TDP expression to reduce plant drought stress, *N. benthamiana* leaves were agroinfiltrated with TDP expression constructs. Expression constructs for each of the five TDP variants in this study were generated using the GoldenBraid 2.0 golden gate cloning strategy, but unfortunately a design flaw led to the TDPs being out-of-frame. While we intended to express TDPs with an N-terminal GFP tag, the TDPs were likely not expressed, although it is possible some TDP fragments may be expressed. Prior to identifying this design flaw, constructs were transformed into *Agrobacterium* strain GV3101. Single *Agrobacterium* colonies from each TDP construct transformation were selected and stocked for transient expression assays in *N. benthamiana* leaves.

DNA Construct Lack GFP Expression

Three leaves, each from two mature *N. benthamiana* plants A and B, were infiltrated with the five different out-of-frame constructs. Younger leaves, lower on the plant, were more receptive to uptake of the agroinfiltration solution syringes. Infiltration into older leaves was more difficult and increased circular scarring around the infiltration sites from the syringe. For three days, the plants were watered routinely and displayed no signs of stress.

After removal from the plant, the three infiltrated leaves and one control leaf were

immediately screened for GFP fluorescence with the same exposure.

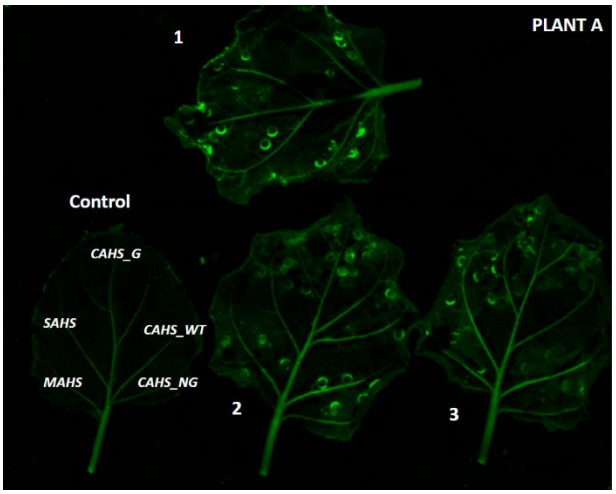

Figure 1. Plant A leaves (bottom view) GFP imaging. The control leaf is bottom left, with trial leaves on right. Infiltration sites: MAHS, SAHS, CAHS gelling (CAHS_G), CAHS wild-type (CAHS_WT), and CAHS non-gelling (CAHS_NG).

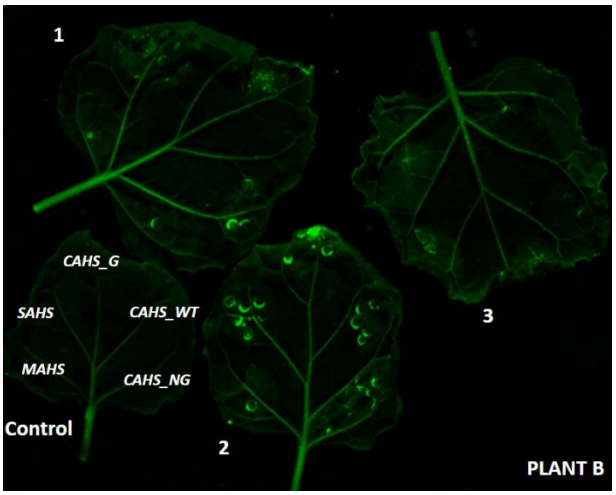

Figure 2. Plant B leaves (bottom view) GFP imaging, with control leaf on bottom left. Infiltration sites: MAHS, SAHS, CAHS gelling (CAHS_G), CAHS wild-type (CAHS_WT), and CAHS non-gelling (CAHS_NG).

The intensity of GFP observed in the leaves was inconsistent and mainly outlined the circular scars from the syringes. As shown in **Figure 2,** there was more green fluorescence concentration where CAHS-G was infiltrated in leaf 2 of Plant B, but this was not consistent across all leaves and potentially due to autofluorescence. This inconsistency suggests that fluorescence may not be due to the presence of fluorescent proteins, which should still be expressed in these constructs. The little fluorescence is most likely due to scarring and the natural green color of the leaves. Regardless, the inability to observe prominent GFP signal in the infiltrated leaves in part led us to identify the error in the TDP constructs.

Osmotic Stress-Induced Luminescence

Due to time constraints, the TDP constructs were utilized in the subsequent osmotic stress test, despite lacking evidence of GFP expression. Twenty leaves across three *N. benthamiana* plants were labeled and prepared for agroinfiltration, four leaves for each of the five constructs. In this test, the effect of the incorrect expression constructs co-infiltrated with the ABA luminescence reporter, P447-FBP-19, was analyzed via *Agrobacterium*-mediated agroinfiltration and simulated drought stress. The production of ABA in response to drought stress causes P447-FBP-19 to produce a luminescence signal[7]. When co-infiltrated with correct TDP expression constructs, the stress-induced luminescence would ideally be mitigated.

Six areas on each leaf were infiltrated using syringes: the specified TDP, TDP co-infiltrated with P447-FBP-19, and three controls, P447-FBP-19 alone, P362-FBP-12 (constitutive luminescent signal), and wild-type GV3101 *Agrobacterium*. After three days of routine watering, the leaves were excised with one replicate left to dry and a second replicate floating in water. Every hour over the course of 12 hours, chemiluminescence and GFP images were taken of the leaves. Images at key time points, hours 4 and 10, were analyzed using ImageJ software, since the greatest luminescent ABA reporter response was recorded at hour 10.

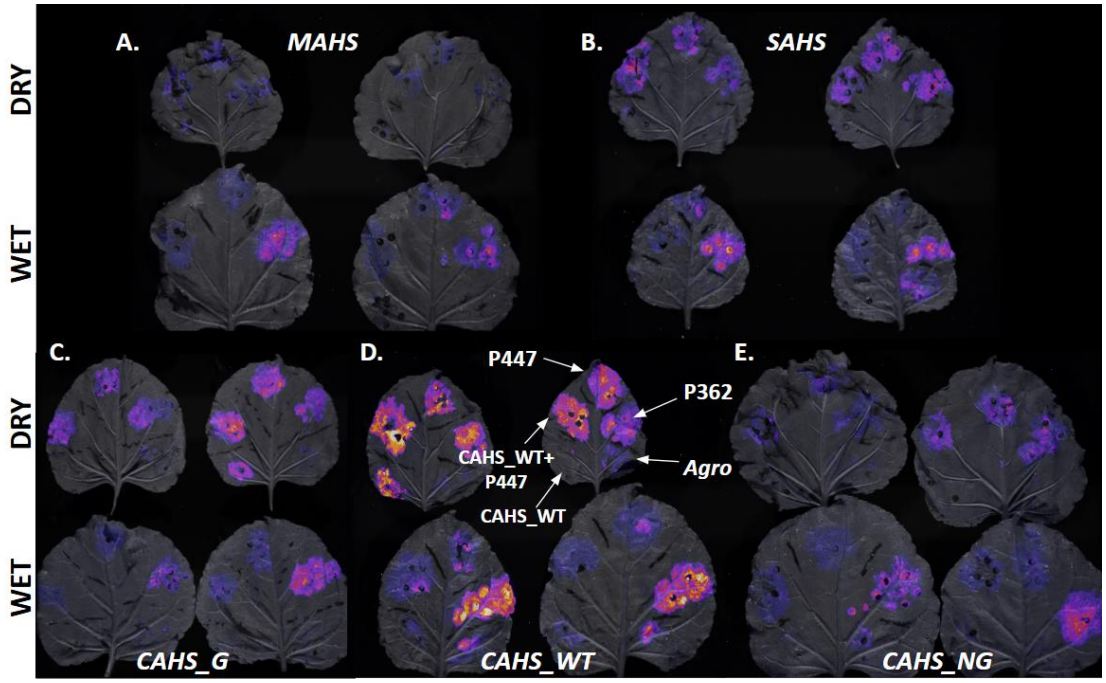

Figure 3. Chemi blots of dry (top row) and wet (bottom row) leaves for infiltrated TDPs and controls, P447, P362, and Agrobacterium. A) MAHS, B) SAHS, C) CAHS Gelling (CAHS_G), D) CAHS wild-type (CAHS_WT), E) CAHS non-gelling (CAHS_NG).

Using the ImageJ ellipse tool (fixed area) and measure function, the degree of luminescence of each infiltrated area was measured over time. Fold changes were calculated by dividing integrated density of dry leaves over that of wet leaves for each CAHS variant at each key timepoint. For the three controls, the averages of the dry data were divided by the wet for all leaves to calculate the average fold changes.

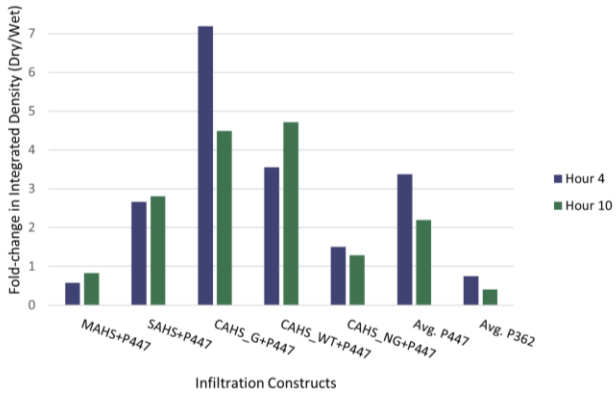

Figure 4. Luminescence fold change of TDP+P447 and controls.

Controls, P362, P447, and wild-type *Agrobacterium*, behaved as expected under osmotic stress. The constitutive P362 luminescent reporter luminesced, regardless of the drought conditions. The wild-type *Agrobacterium* slightly luminesced, potentially due to the bleeding of another infiltrate. As shown in **Figure 3**, the P447 stress reporter luminesced more, or expressed more, in the dry leaves. While we cannot make any conclusions about the effect of TDPs on ABA response in simulated drought stress due to the incorrect TDP expression cassettes, this data does support that this transient infiltration and simulated drought stress method could be used to measure this effect with some additional optimization to minimize variation.

However, we did observe some variation in ABA responsive luminescence between constructs and across time. CAHS_G+P447, and CAHS_WT+P447 co-infiltrates showed higher luminescence than the P447 alone, meaning CAHS gelling and CAHS wild-type did not reduce P447

expression, indicating increased ABA signaling. Though MAHS+P447 fold change was less than that of P447 alone, the fold change increased over time (**Figure 4**). Positive fold change values indicate that protein expression is upregulated, and luminescence is higher in the dry (stress) conditions compared to wet conditions. So, MAHS+P447 luminescence or ABA response was upregulated in dry conditions compared to wet overtime. Similarly, the fold change of SAHS+P447 increased slightly over time, showing an increase in stress response. CAHS_NG+P447 luminescence was also less than that of P447 in the dry leaves of CAHS non-gelling. This variation may have been due to differences in leaves and plants, and therefore including control construct infiltrations on every leaf that can be used to normalize luminescent values and control for this variation is very important in future experiments.

Troubleshooting TDP Constructs

The inconsistency in these experiments led us to identify that the TDP plasmid constructs were incorrectly designed. With the addition of the mVenus reporter tag, GFP signal should have been visible in **Figures 1 and 2**. Lack of GFP expression suggests that mVenus is not expressing correctly. This is possibly due to nonsense-mediated RNA decay because of the early stop codon resulting from the frameshift in the TDP sequence[9]. Thus, successful introduction of TDPs to the plant vector via agroinfiltration cannot be confirmed.

Analysis of the TDP assembly constructs in Benchling revealed that the absence of two guanine bases, gg, caused the overhangs to misalign. This contributed to the failure of mVenus as the reporter tag did not properly integrate into the plasmid structure and transcribed during DNA replication.

Experimental Methods

TDP Gene Synthesis

Tardigrade protein sequences were obtained from Dr. Thomas Boothby, of the University of Wyoming. Dr. Boothby provided sequences for five different TDPs: MAHS, SAHS, CAHS wild-type, CAHS-gelling, and CAHS non-gelling. The amino acid sequences were then uploaded to Twist Biosciences and ordered, along with the sequence for the mVenus GFP reporter. The TDPs and mVenus from Twist were diluted with water, according to their reported yield (ng) to ensure all synthesized parts were roughly equal in concentration.

GoldenBraid: Level 0 Assembly

The GoldenBraid 2.0 DNA assembly framework was used for development of DNA constructs. To integrate the synthesized TDPs into DNA constructs, the Level 0 entry vector pUPD2 and binary vector PDGB3 (backbone) were inoculated for preparation of Level 0 assemblies[14]. With the GoldenBraid kit over ice, Lysogeny Broth (LB) growth media was pipetted into two sterile test tubes, labelled pUPD2 and PDGB3. Then, respective antibiotics for each vector were added to the properly labelled tubes: chloramphenicol (50 mg/mL) for pUPD2 and kanamycin (50 mg/mL) for PDGB3. A pipette tip was used to collect a single ice crystal from the respective well from the GoldenBraid kit and swirled into the correctly labelled tube. Tubes were left to incubate overnight at 37°C at 250 rpm. Miniprep of DNA was conducted using Monarch® kits[1].

For the Level 0 assembly, TDPs and mVenus were digested using BsmBI restriction enzymes. The assemblies were then transformed into *E. Coli* Top 10 competent cells with respective DNA added into labelled 1.5 mL tubes. The DNA tubes were heat shocked at 42°C for 30 seconds and then incubated for an hour at 37°C. The assemblies were then plated on six labelled

chloramphenicol, IPTG, and X-gal plates for blue-white screening of colonies. Transformation of Level 0 assemblies was successful, with multiple white colonies present on plates after 24 hours. Colonies from each plate were inoculated and left to incubate overnight at 37°C.

GoldenBraid: Level 1 Assembly

Promoter, pP2x35, and terminator, TatHSP, were inoculated from the GoldenBraid kit with ampicillin antibiotic for preparation of Level 1 assemblies[14]. The assemblies, containing PDGB3 (backbone), pP2x35, TatHSP, specific TDP, were then placed in the thermocycler for the BsaI assembly protocol. Using five tubes of *E. Coli* competent cells, the Level 1 assemblies were transformed and plated on kanamycin plates for incubation overnight at 37°C. The next day, two colonies were collected from each plate and inoculated in separate tubes for overnight growth, labelled TDP 1 & 2.

To test viability of the Level 1 assemblies, a linear digest with BsmBI restriction enzyme was conducted. For each PCR tube the contents were 2.5 µL NE Buffer, 1 µL BsmBI, 16.5 µL water, and 5 µL DNA for a total volume of 25 µL in each tube. While in the thermocycler, gel electrophoresis was prepared for analysis of assemblies. Based on imaging of the gel and assembly concentrations, the assemblies sent to Plasmidsaurus for sequencing were MAHS1, SAHS2, CAHS_G2, CAHS_WT1, and CAHS_NG2 (see **Appendix)**.

Transformation in *Agrobacterium*

The Level 1 assemblies were then transformed into *Agrobacterium* to mediate agroinfiltration. *Agrobacterium* competent cells were combined with TDP DNA and subjected to electroporation, according to protocol[8]. Then, the transformants were incubated on a roller drum at 30°C for 2 hours. Each TDP transformation was plated and incubated for 48 hours at 28°C. The next day, the colonies were inoculated in LB media, with equal amounts of kanamycin and rifamycin, and left to grow overnight at 28°C.

Agroinfiltration of *N. benthamiana*

Infiltration Media

To create sterile infiltration media, 250 mg D-glucose, 5 mL MES solution, and 5 mL Na_3PO_4-$12H_2O$ stock, 250 µL acetosyringone, 50 µL 10% tween, and water to yield 50 mL total volume were combined in a falcon tube.

Preparation and dilution

Two tubes per construct, with 1 mL of inoculated transformations per tube, were centrifuged for 5 minutes. The supernatant was then removed and 1 mL of infiltration media added to resuspend, repeated twice.

The construct concentrations were measured with cuvettes in the Nanodrop. Dilutions with the infiltration media were performed to yield desired OD of 0.8 – 1.0 after initial 1:5 dilution (200 µL construct: 800 µL infiltration media). The following recovery step required the DNA infiltrates to shake in the incubator for at least 4 hours. After the recovery step, plastic syringes were used to push about 0.1 mL of each TDP construct into the labelled areas on the leaves, though volume varied depending on effectiveness of DNA uptake by the leaf. Three days would elapse before excision from the plant for testing to ensure DNA could be replicated within the plant vector.

Osmotic Stress Testing

Preparing Controls

Three controls had to be prepared prior to osmotic stress testing: P362, P447, wild-type *Agrobacterium*. The constitutive luminescent reporter, P362, was grown by inoculating an ice chip from its toolkit into 100 µL of LB media. High and low concentrations were plated on kanamycin plates. The P447 biosensor, stress-induced luminescent reporter, was transformed into *Agrobacterium* via the previously defined

procedure, and 100 µL plated on kanamycin plates. Then, wild-type *Agrobacterium* was transformed by skimming off 5 µL of *Agrobacterium* competent cells and diluting with 95 µL LB media. High and low concentrations were plated on LB media plates. All three controls were grown at 28°C for two days. Next, colonies of each control and the five TDPs were inoculated in different tubes with 5 mL LB media, 5 µL kanamycin, and 5 µL rifampicin, except for the wild-type *Agro* which did not receive kanamycin. The eight tubes were grown overnight in a shaking incubator at 28°C.

Agroinfiltration & Imaging

Across several *N. benthamiana* plants, 20 leaves were labelled and differentiated with colored tape around the stems, four leaves per TDP. On each leaf, 6 sections were labelled for each infiltrate. The same agroinfiltration procedure was followed to prepare the controls and TDPs[8]. Equal amounts of diluted P447 were combined into single tubes for co-infiltrations with TDPs (~1 mL each). Syringes were used to infiltrate with at least 0.1 mL into each labelled section, ensuring no bleeding over from the six infiltrates on each leaf.

After three days of normal watering, the 20 infiltrated leaves were excised. Half of the leaves were placed in individual pre-labelled square petri dishes to dry out and the rest were placed in petri dishes with water. Over the course of 12 hours, an iBright FL1500 fluorescent/luminescent imager (Thermofisher) was used to take GFP and Chemi luminescent blot images of the leaves, each image showing the four leaves (wet & dry) per TDP. See **Appendix** for procedure pictures.

Conclusion

This study successfully established a procedure to introduce tardigrade protein constructs and ABA stress-responsive controls into a plant vector via agroinfiltration in *N. benthamiana*. The ultimate objective of the study was to analyze the effects of different TDP constructs in reducing plant drought stress responses and improving tolerance for use in crop cultivation on the ISS. However, due to errors in the sequencing of the tardigrade DNA constructs, we could not assess the ability of TPDs to confer plants with drought tolerance. Despite this, the experimental assays to test osmotic stress after agroinfiltration proved to still be efficient and should be repeated with correct DNA constructs and additional per leaf normalization.

Future Work

In addition to repetition of agroinfiltration in *N. benthamiana*, another method to introduce T-DNA plasmids into *A. thaliana* is floral dip of mature flowers into prepared infiltrations[16]. The same procedure for dilution and recovery step with infiltration media of the TDP infiltrates would be utilized. Mature flowers should be labelled with color coded tape to differentiate TDPs. Pipettes with enough TDP constructs can be used to submerge respective mature flowers. In a few weeks, this method would produce transgenic seeds which could then be grown to yield mature plants naturally tolerant to desiccation. This method could be used to grow crops or prepare transgenic seeds that are pre-conditioned to be more tolerant against desiccation prior to transportation to the ISS. With this advancement in crop growth, a greater variety of vegetables and fruits can be grown in support of crop cultivation in outer space.

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Appendix

Level 1 Assembly Gel Electrophoresis

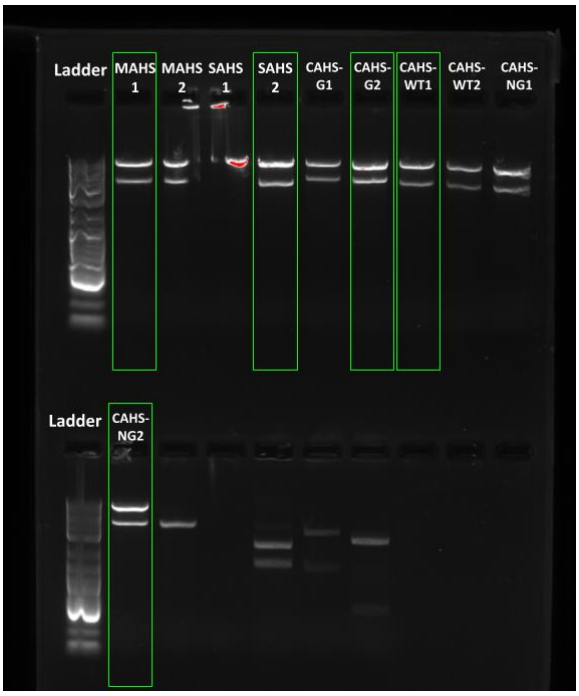

Figure A1. Selection of Level 1 Assemblies using Gel Electrophoresis.

Agroinfiltration & Osmotic Stress Test

Figure A2. Agroinfiltration with syringe (top left), Drying leaves (top right), Floating leaves in dishes (bottom).

Osmotic Stress Testing: GFP Images at Hour 10

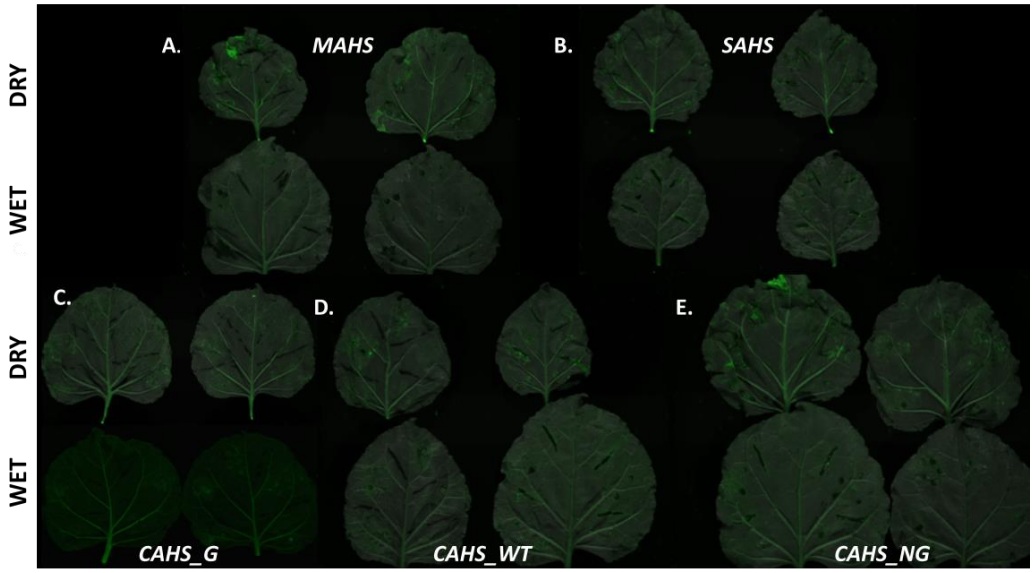

Figure A3. GFP expression in leaves agroinfiltrated with MAHS, SAHS, CAHS gelling (CAHS_G), CAHS wild-type (CAHS_WT), and CAHS non-gelling (CAHS_NG).