UNIVERSITY OF CALIFORNIA
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Modulation of ABA Receptor Function

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Plant Biology

by

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CHAPTER 1

ABSTRACT

Abscisic acid (ABA) is a phytohormone that plays important roles in plant development and environmental stress response. When bound to a family of star-related lipid-transfer (START) proteins, clade A subfamily of type II C protein phosphatases (PP2Cs) are negatively regulated leading to ABA response. It was only recently that the mechanism involved in ABA perception was identified. This was due in part to the success of bypassing this redundancy with the use of forward chemical genetics in *Arabidopsis thaliana.* Many biological findings utilizing forward chemical genetics have been limited to *A. thaliana.* Although a general insight has been gained from these studies, *A. thaliana* cannot fully address development and physiological phenomena in monocots. Here we describe a screening system in *Panicum virgatum* (Switchgrass) which we developed and used for a forward chemical genetics screen in search of an ABA agonist exclusive to monocots. Although a bonafide agonist was not isolated, our findings do illustrate the potential biological studies our screening system can be extended towards.

INTRODUCTION

Abscisic acid (ABA) is an essential phytohormone involved in plant development and abiotic stress response (Finkelstein, 2002). *In vivo,* ABA binds to a family of star-related lipid-transfer (START) proteins that, in their active state, inhibit members of the clade A subfamily of type II C protein phosphatases (PP2Cs). Inhibition of PP2Cs leads to the activation of sucrose non-fermenting-1 (SNF1) -related protein kinase 2 (SnRK2) kinases

which results in phosphorylation of downstream targets responsible for ABA response. Much focus had been placed on dissecting its mechanism of action since ABA's discovery in the 1960s (Park, *et al.* 2009). One challenge faced was bypassing the genetic redundancy of the START protein family. This is was overcome with the introduction of chemical genetics.

The use of small molecules to identify proteins regulating specific phenotypes has been termed forward-chemical genetics (Stockwell, 2000). The use of chemical genetics has grown over time, in part because it enables one to dissect pathways spatially and temporally without relying on mutagenesis (Stockwell, 2000). In plants, genetic redundancy is a common obstacle to overcome when it comes to dissecting biological processes. Many biological pathways consist of complex networks that may contain families of genes that are redundant in function. A problem with the use of mutations which result in loss-of-function and gain-of-function is that they are not conditional and for many gene families, they cannot completely be turned off or on at will due to redundancy. In plants, polyploidization is a common mechanism of gene duplication resulting in genetic redundancy. About 70-80% of angiosperms have gone through polyploidization, with at least four whole-genome duplication events occurring in *Arabidopsis thaliana* (Liu, *et al* 2008). In *A. thaliana* two-thirds of its genes are known to have at least one homolog within the genome; with 37.4% of its proteins belonging to families of more than five members (Arabidopsis Genome Initiative). The chemical genetic approach can be one tool used to bypassing genetic redundancy by using small

molecules to perturb protein function in a rapid, conditional, and specific manner (Stockwell, 2000).

For example, Park *et al* 2009 were able to overcome genetic redundancy using forward-chemical genetics to identify Abscisic Acid (ABA) receptors in *A. thaliana*. The ABA pathway plays key roles in plant development, drought tolerance, and other abiotic stress response (Danquah, *et al*. 2013). Park, *et al.* 2009 relied upon a general three-step workflow that entails: 1.The assembly of small molecules; 2.High-throughput screening for phenotypes of interest (such as germination inhibition); and 3. Identification of ligand target(s) (Stockwell, 2000). With this approach, Park *et al.* isolated Pyrabactin, a selective ABA agonist that helped identify the receptors involved in ABA signaling modulation. Due to the genetic redundancy of the START protein family, single-locus mutations were not a reliable method to dissecting their function. Microarray analyses of ABA and Pyrabactin response in seeds and seedlings revealed that both induced similar transcriptional responses. Further investigation of pyrabactin's specific target, PYR1, revealed that 13 genes in *A. thaliana* shared much similarities in their amino acid sequences and overlapping expression patterns (Park, *et al* 2009).

The discovery and knowledge of many developmental and environmental stress response pathways known to plants, including the biosynthesis and response to ABA, have mainly been conducted in *A. thaliana*, a dicot species. Significant research has revolved around this reference species with the notion that this knowledge can be translatable to higher plant species and monocots, in particular staple crops (Varshney, *et al.*2010). With this in

mind, differences in genetic and physiological response pathways vary among species especially between dicots and monocots (Kim, *et al.* 2011). Although a general knowledge of these pathways is understood, much of which has resulted from methods such as forward chemical genetics, *A. thaliana* cannot fully address insights pertaining to monocot development and physiology under stress and non-stress conditions.

Because many of the world's major staple crops are monocots, notably grasses, efficient grass model systems are needed. *Oryza sativa* (rice) became the first genomics model for representing monocot systems due to its small genome size, its slow generation time and large size are problematic from the perspective of chemical genetics (Varshney, *et al.* 2010). Although research on a number of other model species, such as sorghum (*Sorghum bicolor*), wheat *(Triticum* spp.), and barely (*Hordeum vulgare*), have gained focus, the use of forward-chemical genetics in monocots has not been reported (Strable, *et al.* 2009). The large seed size of rice, maize and most other staple crops makes it more difficult and expensive to conduct high throughput phenotype-based screens. There is therefore a need for a monocot model for use in phenotype-based chemical genetic screens.

Here we describe a screening system using *Panicum virgatum* (Switchgrass), a perennial grass species that shares high levels of synteny with grass genomes such as sorghum, rice, and maize (Sharma *et al.* 2012). *P. virgatum* was used as it met many of the similar selection criteria that had made *A. thaliana* an ideal model for biological studies: a fully sequenced genome, short life cycle from germination to maturation, and commercial

availability (Meinke, *et al.* 1998). *P. virgatum* has a haploid genome size of approximately 1.5 Gb and produces a small seed that makes it easy to handle and process (Casler *et al.* 2011). Today, it has also become a significant source of lignocellulosic biomass for biofuels. Production of perennial grasses for biofuels is expected to take place on marginal soils that are not ideal for conventional row crops. Evidently, these soils may likely be subjected to drought frequently (Jiang, *et al.*2012). The goal of the work described in this chapter was to (A) develop the Panicum screening methodology as a platform for chemical genetic screens in a monocotyledous (B) our chemical screen was to identify an ABA agonist(s) with bioactivity in monocots.

RESULTS

Designing a high-throughput screening system for Panicum virgatum

High-throughput screening has become a fundamental component of chemical genetics. In *A. thaliana*, a variety of novel protocols has been well documented. Unfortunately, methods in preparation of high-throughput screening such as seed sterilization and efficient seed distribution for *P. virgatum* have not been well established. Another factor that had to be considered was in regards to the concentration of ABA that would result inhibition of seed germination in *P. virgatum*. This was addressed by performing a dose curve to determine the minimum concentration that would produce the phenotype of interest.

Preliminary sterilization techniques investigated involved modified protocol from *A. thaliana* such as gas and liquid chemical sterilization (Data not shown). Our goal was to identify an effective method that not only prevented contamination but did not affect germination and growth. Surface sterilization with Cl_2 gas for 10 or more hours inhibited or delayed germination severely and was unable to keep conditions sterile. The use of ethanol and bleach with 10% Tween at a variety of different concentrations and wash times produced similar results. An underlying issue with prolonged bleach exposure was the whitening of seeds that would result in complete inhibition of germination. We then came across a technique in *Oryza sativa* that used sulfuric acid (H_2SO_4) for seed scarification prior to sterilization. The most effective technique we came across involved 96% H_2SO_4 solution in water (Acros Organics) for 5 minutes, washing with ddH_2O, placing the seeds in 5.25% hypochlorite bleach plus 10% Tween-20 solution in 100;1 ratio, and rinsing in ddH_2O to remove any bleach solution (See Materials and Methods). In combination with liquid chemical sterilization, we were able to develop an effective method that kept *P. virgatum* sterile without affecting germination and growth.

In *A. thaliana*, seeds are commonly distributed in a high-throughput manner with the use of 0.1% liquid agar. Compared to *A. thaliana* seeds (0.5m,m), *P. virgatum* seeds (2-3mm) are much larger (Meinke, 2010. Oklahoma State University, 2013). In 0.1% liquid agar, *P. virgatum* was much denser than *A. thaliana* and could not be easily taken up by pipetting. To overcome this, we dried sterilized seeds and used a half-skirted, rimmed 96-well PCR plate containing 1% Agarose to transfer them onto chemical plates. The result we found was that a similar amount of seeds would be transferred to each well.

Identification of a potent Panicum seed germination inhibitor

A library of ~20,000 sulfonamide compounds, 9807 from Chembridge (www.chembridge.com) and 9995 from Enamine (www.enamine.net), dissolved in DMSO to a concentration of 100 µM, were screened in duplicate for their ability to inhibit seed germination in *Panicum virgatum*. *P. virgatum* seeds were sterilized and placed in wells containing 1-µL of compound, 1/2 x Murashige&Skoog (MS), and 0.7% agar. Plates were stratified in 4°C for 3 days, transferred to a dark humidity chamber regulated at 95% Relative Humidity for 4 days, then scored. Initial screening identified 100 hit wells. Our high false positive rate is likely a consequence of asynchronous germination of Panicum seed which leads to occasional wells in which no seeds have germinated. This can be addressed by examine replicate data, however we chose to conservatively retest all potential hit molecules. A total of 14 compounds (Figure 1.1A, 1.1B) inhibited germination at 100 µM and were therefore selected for further characterization of potential agonist activity (Figure 1.2).

In *P. virgatum*, 4 compounds were able to inhibit germination at 100 µM. *P. virgatum* seeds were sterilized and sewn onto plates containing 25µM or 100µM chemical respectively, 1/2x MS, 0.7% agar, stratified in dark at 4°C for 3 days, and scored after 4 days at 25^{o}C, 16 h light, 8 h dark. A sulfonamide we named EM51D05, inhibited *P. virgatum* seed germination at concentrations as low as 25 µM. The three other compounds, SA22B11, SA41D09, and EM116G02 are also bioactive at 25 µM, but not to the extent of EM51D05 (Figure 1.2). *P. virgatum* germination was also inhibited at 25

µM ABA. The remaining 10 hits were not characterized further due to their weak activity at 25 µM. Bioactivity was also investigated in *A. thaliana.* Seeds of wildtype Col-0 and ABA insensitive mutant *abi1-1* were screened against our original 14 bioactive hits. An ABA agonist might inhibit seed germination in *Arabidopsis thaliana,* so this was tested. 2 of the compounds, SA22B11 and SA41D09, delayed Arabidopsis seed germination at 25 µM, but this effect could not be abolished by the dominant abi1-1 mutations (Figure 1.3) suggesting that the effects of these compounds are not likely to act through the ABA signaling pathway in Arabidopsis. These findings suggest that the specificity of these bioactive compounds is limited to *P. virgatum*. Further demonstration of this specificity is observed at concentrations as low as 1 µM. Three days after germination, the remaining 10 hits that were not characterized further were able to delay germination at lower concentrations (Figure 1.4). Thus, our screening efforts identified 4 inhibitors of Panicum seed germination.

Physiological characterization of hit compounds

One of the well-characterized effects of ABA is to inhibit root growth. We therefore tested the effects of the 4 bioactive hits on root growth in comparison to ABA. *P. virgatum* seeds were sterilized and sewn onto 1/2x MS, 0.7% agar gridded plates which were sealed with 3M Micropore Paper Tape. After stratification and germination, seedlings that were 1 cm in length were transferred onto gridded plates containing similar media containing the respective concentration of compound. Root length was quantified

after 5 days. At 100 µM, all compounds were able to inhibit Panicum root growth to a similar degree as observed with100 µM ABA (Figure 1.5). At 25 µM, all hits inhibited root growth similarly or better than ABA at the same concentration. Interestingly, EM51D05 also affected growth of above ground structures at 25 µM and 100 µM. In ABA-treated control plants, this phenotype is only observed at 100 µM ABA.

To further characterize the activities of the 4 hits in vegetative tissue, we conducted drought stress assays on mature plants. 30 day old plants were sprayed (every other day) with 50 µM and 100 µM compound during a 7-day water deprivation period. Total biomass was measured to infer the effects of drought. These experiments showed that after 3 treatments during the7 day period of limited watering, ABA treated plants outperformed all treatments at both 50 µM and 100 µM concentrations (Figure 1.6). After rewatering, ABA treated plants displayed more pronounced recovery compared to DMSO and the 4 bioactive chemical treatments. These experiments suggest that the compounds are not capable of inducing drought tolerance in *Panicum virgatum*.

Receptor analyses

To further characterize the activity of our bioactive hits, we examined their ability to activate different ABA receptors obtained from *Zea mays* and *A. thaliana* (AtPYR1, AtPYL2, AtPYL5, AtPYL8, and AtPYL12) using yeast two-hybrid reporter strains (Figure 1.7). Clones for *Zea mays* ABA receptors were obtained from Syngenta (www.syngenta.com). All receptors reported activation at 5 µM and 25 µM ABA however at1 µM ABA, receptors g, AtPYL8, and AtPYL12 did not report activation.

Receptor activation in the presence of pyrabactin was observed in receptors i, j, k, AtPYR1, and AtPYL2. These control experiments demonstrate that the yeast assays were behaving as expected. EM30G11 was only able to activate AtPYL5 weakly at 25 µM. SA98F08 was only able to activate AtPYL8 weakly at 25 µM. The remaining hits were unable to activate any receptors obtained from *Z. mays* and *A. thaliana*. Given the high sequence conservation of residues in the ABA binding pocket of the ABA receptors, these results suggest that the hit molecules are not likely to activate ABA receptors. However it is possible that the metabolism, uptake or excretion of the compounds in yeast is different than in Panicum. It is also possible that Panicum receptors could respond to the compounds tested, however we did not test this.

Characterization of ABA responsive marker genes in Panicum

Given the effects of our bioactive molecules on germination, root growth, and abiotic stress, we wanted to see if they affected ABA-regulated gene expression using quantitative RT-PCR analyses. Despite their inability to activate receptors in the yeast assays, we wanted to investigate if the compounds activate the ABA pathway, as measured using Panicum ABA-responsive marker genes.19 well characterized ABA responsive genes were selected and their sequences used for BLAST searches against all green plant proteins found in the NCBI Database to identify Panicum homologs. Based on these analyses, primers were designed for 19 *P. virgatum genes* (Figure 1.8).These primers were then used for qRT-PCR experiments. 1 week old *P. virgatum* seedlings

were incubated in liquid media containing DMSO or ABA. cDNA was isolated 24 hours after incubation. These analyses showed that 2 genes, *pvCOR47* and *pvABI1*, demonstrated significant induction by ABA (compared to Actin control) and these were therefore used as molecular markers for characterizing ABA responses in Panicum. (Figure 1.9, left). Our results showed that ABA produced an ~9-fold induction of *pvCOR47* after 1 hour of treatment (Figure 1.9, right). 24 hours after treatment, *COR47* was induced ~278-fold. The 4 bioactive hits produced less than a 2-fold induction of *COR47* at both 1 hour and 24 hour treatments. With the *pvABI1*probeset, a ~6-fold induction is observed in ABA treatments at 1 hour and 24 hours. The 4 bioactive hits produced less than a 1.5-fold induction of *ABI1* at both 1 hour and 24 hour treatments. These experiments suggest that the compounds identified do not activate the Panicum ABA- response pathway. We have successfully created 2 useful qRT-PCR markers for characterizing ABA responses in Panicum, which will be valuable for future characterization of ABA signaling in Panicum.

DISCUSSION

In this report, we demonstrate a chemical screening system for monocots using *P. virgatum* as the model organism. A secondary goal of this screen was to identify an ABA agonist active in monocots. There were several criteria in organism selection that had to be met in order for an efficient experimental system to be developed. Much of these were based off qualities *A. thaliana* possessed. First, *A. thaliana* has a fully sequenced and

annotated genome. It has a relatively short life cycle of six weeks, from germination to seed maturation, and can easily be propagated in small spaces (Meinke *et al.* 1998). For high-throughput screening purposes, *A. thaliana*'s seed size is quite small. We ideally wanted a monocot that was commercially available and had a fully sequenced genome to conduct molecular and genetic analyses. Seed size was an important consideration as it could potentially influence the amount of chemical ligand needed to produce a notable phenotype. Having a library of ~20,000 small molecules, quantity was limited. We selected *P. virgatum* because it fulfilled many of the qualities noted by Meinke *et al.* 1998 that made *A. thaliana* an ideal model for high-throughput screening. It was also selected due to its agronomic impact to society and its growing interest towards production for biofuels.

The next challenge faced was developing an efficient high-throughput screening system that could be applied to *P. virgatum*. There is no previously established protocol for chemical screens in monocot systems. One of the constraints was seed size. *P. virgatum* seeds are larger than *A. thaliana* but significantly smaller than *Z. mayes*. This would still allow the use of a modified protocol for a 96-well high-throughput screening system done on *A. thaliana*. Our next challenge to overcome was seed sterilization methodology as no previous protocol for *P. virgatum* had been developed. A variety of different surface sterilization methods were performed until we determined an ideal method that avoided contamination and did not affect seed germination. A distribution system also had to be developed due to *P. virgatum*'s seed size as conventional pipetting of seeds suspended in

liquid agar was unachievable. After much trial and error, an optimized high-throughput screening system for *P. virgatum* was finalized.

The library used for the high-throughput forward genetic screen consisted of structurally diverse compounds that each contained a sulfonamide linkage, one of the bioactive functional groups of pyrabactin. Preliminary screening uncovered a handful of bioactive hits that were later narrowed down to 14 candidates for further characterization of potential agonist activity. From our germination and root growth assays, only 4 candidates of the 14 isolated compounds were active at 25 µM concentrations. Our strongest candidate, EM51D05, produced similar phenotypes as observed in the presence of ABA at similar concentrations. Although compared to ABA, EM51D05 at 25 µM had a stronger affect on both root and shoot growth. In the Yeast Two-Hybrid screen of *A. thaliana* and *Z. mayes* ABA receptors, only two compounds from the original 14 were able to weakly activate two receptors. This suggested that the targets of our candidate compounds were downstream of perception or unrelated to the ABA pathway altogether. To investigate if these compounds do in fact activate the ABA pathway, we focused our attention directly towards the genes responsible for eliciting ABA response. Quantitative RT-PCR was conducted on ABA responsive genes conserved in both *A. thaliana* and *P. virgatum*. From the 19 genes characterized, *pvCOR47* and *pvABI1* demonstrated significant induction by ABA (compared to Actin control) and were used as molecular markers to monitor ABA response in *P. virgatum*. At both 1 hour and 24 hour treatments, none of the 4 bioactive hits were able to produce similar or greater induction compared to

ABA. These findings suggest that the compounds identified do not activate the ABA-response pathway in *P. virgatum*.

While we were unable to find a bona fide ABA agonist, we hope to be able to screen other chemical libraries. Our work showed that we were able to develop a high-throughput screening method that can potentially be adapted towards other monocot systems. With this, we hope that the knowledge gained from this study can increase flow of knowledge and technology in order to improve crop production for agriculture and biofuels production.

MATERIALS AND METHODS

Plant material and growth conditions

Panicum virgatum seeds (Wildseed Farms, Item# 3809. 86% Purity, 73% Germination) were sterilized in 96% H_2SO_4solution in water (Acros Organics) for 5 minutes then rinsed twice with ddH_2O, each 5 minutes. Seeds were then transferred to 50ml centrifuge tube. 5.25% hypochlorite bleach plus 10% Tween-20 solution in 100;1 ratio is added to the centrifuge tube and repeatedly inverted for 45 minutes. This solution was then decanted and the seeds are washed with sterile ddH_2O six times, each rinse being 5 minutes. Rinsed seeds are then placed onto filter paper and dried in the hood. Seeds were then sewn on *1/2*x Murashige&Skoog (MS), 0.7% agar plates and stratified in dark at 4°C for 3 days. For germination assays, seeds were sewn onto plates containing 25µM or

100µM chemical respectively. Equivalent volumes of DMSO were added to plates as control. For root and qRT-PCR assays, seeds were sewn onto *1/2*x MS, 0.7% agar gridded plates and sealed with 3M Micropore Paper Tape. After stratification, plates are grown in chambers set at 25°C, 16 h light, 8 h dark. For root and qRT-PCR assays, plates are grown vertically. Seedlings that are 1 cm in length are transferred to *1/2*x MS, 0.7% agar gridded plates containing 25µM or 100µM chemical respectively and grown vertically for 5 days. For qRT-PCR assays, 4 day old seedlings are transferred to 250 ml flasks containing 5ml *1/2*x MS, 0.5% Sucrose liquid media and grown for 7 days. Media is then replaced with 5ml *1/2*x MS, 0.5% Sucrose liquid media containing 25 µM and 100 µM of chemical respectively and grown for indicated time periods. Equivalent volumes of DMSO were added to plates and flasks as control.

Chemicals

A collection of 9807 and 9995 sulfonamide analog compounds in 96-well plates were purchased from ChemBridge and Enamine respectively (www.chembridge.com, www.enamine.net). Compounds were diluted to 10mM in DMSO and working plates were aliquoted to 2.5mM using the Beckman Coulter Biomek FXP Laboratory Automation Workstation. (+)-cis trans Abscisic Acid (BIOSYNTH, A-0120) was diluted to appropriate concentrations in DMSO.

High-throughput chemical screening

Chemical screening plates were prepared containing 1-µL of 10mM compound plus 100-µL 1/2 x MS, 0.7% agar. Prior to addition of 1/2 x MS, 0.7% agar, chemicals were aliquoted into methanol solution using the Beckman Coulter Biomek FXP Laboratory Automation Workstation to ensure transfer. Methanol is allowed to volatilize. To distribute seeds to plates, a half-skirted, rimmed 96-well PCR plate containing 1% Agarose and a 96 Deep-Well plate containing seeds is used. The PCR plate is placed over the Deep-Well plate and inverted to allow seeds to contact the media. The PCR plate is then compressed over the chemical plate and slammed against a surface in order to transfer the seeds. About 5-6 seeds are distributed to each well. Plates are stratified in dark at 4°C for 3 days then transferred to a dark humidity chamber for 4 days. Seed germination is scored by coleoptile emergence.

Determination of root growth

Seeds were sewn onto *1/2*x MS, 0.7% agar gridded plates and sealed with 3M Micropore Paper Tape. After stratification, plates are grown vertically in chambers set at 25^{o}C, 16 h light, 8 h dark. Seedlings that are 1 cm in length are transferred to *1/2*x MS, 0.7% agar gridded plates containing 25µM or 100µM chemical respectively and grown vertically for 5 days. Plates were scanned using an EPSON Perfection 4990. Root lengths were traced using ImageJ 1.45 (Rasband W. National Institutes of Health, USA). Root length is represented by the average percentage grown of all replicates per treatment.

PCR primers

A group of ABA responsive genes in *A. thaliana* were selected from a list provided by Gonzalez-Guzman, *et al.* 2012 for primer design. A BLAST search was performed for conserved proteins against all green plant proteins found in the NCBI database. Conserved regions of the proteins were taken and BLAST searched against the proteome of *Panicum virgatum* (www.Phytozome.com). Contiguous sequences were selected and primers were designed using the Roche Universal ProbeLibrary Assay Design Center (Roche Applied Science). cDNA sequences were also BLAST searched against *P. virgatum*. PCR primers and sequences used for qRT PCR are listed in Table 1.

RNA isolation and Quantiative Real-time PCR

Root and shoot tissue of seedlings were harvested at 1 hour and 24 hour time points, DMSO being t_0. RNA was extracted with the PureLink Plant RNA Reagent according to the manufacturer's instructions (Invitrogen). RNA was purified using the TURBO DNA-*free*™ Kit (Invitrogen). cDNA synthesis was performed using a *SuperScript*™ *III* Reverse Transcriptase (Invitrogen, http://www.invitrogen.com) from a reaction mixture of 5-µg of total RNA, oligo-dT_{20}, and ribosomal RNA primer.15-µL samples for qRT-PCR analysis were prepared by mixing 1.5-µL of cDNA template with Maxima SYBR Green/Fluorescein qPCR Master Mix (Fermentas).qRT-PCR assays were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) in biological triplicate and

triple technical replicate measurements were conducted. Data was processed using the BioRad CFX Manager software. The following conditions were used for each qRT-PCR assay: 3 minutes at 95^{o}C, followed by 40 cycles of 10 seconds at 95^{o}C, and 30 seconds at 55^{o}C in 96-well optical reaction plates (BioRad). After 40 cycles, amplicon specificity was verified by melting curve analysis (65-95^{o}C) and were normalized by PEX4 (T5G25760) internal control.

Drought Assay

P. virgatum seeds are sewn onto soil and stratified in dark for 3 days. Seeds were then grown at 25^{o}C, 16 h light, 8 h dark for 2.5 weeks. Anderson 2.5 inch Band Pot's **(**McCONKEY company) containing 15.5g of dry soil are hydrated. Nine 2.5 week old seedlings are then transferred to these soil pots and grown for 2 weeks. Mature plants are then sprayed with respective compounds at 25 µM and 100 µM concentrations every two days while still being watered. After the third spray, water supply is limited completely. Chemical is sprayed again on the fourth spray cycle. Seven days after watering has been stopped, water is reintroduced. Plant fitness is measured two days after rewatering.

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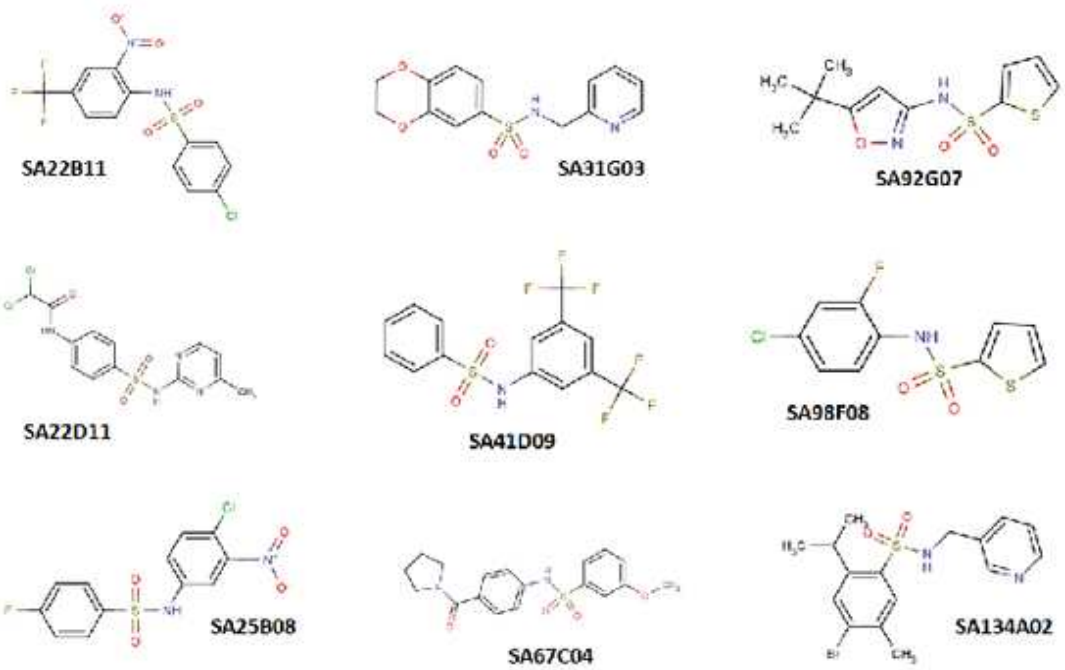

Figure 1.1A. Chemical structure of sulfonamide hits from the ChemBridge library (www.chembridge.com).

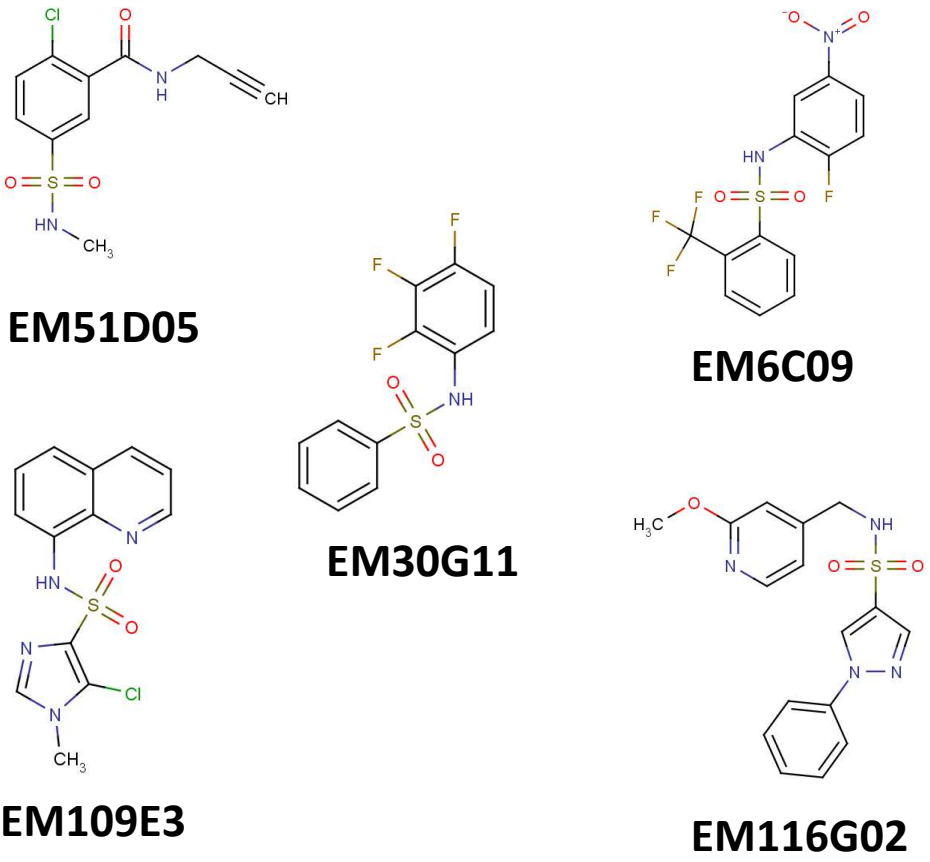

Figure 1.1B. Chemical structures of sulfonamide hits from the Enamine library (www.enamine.net).

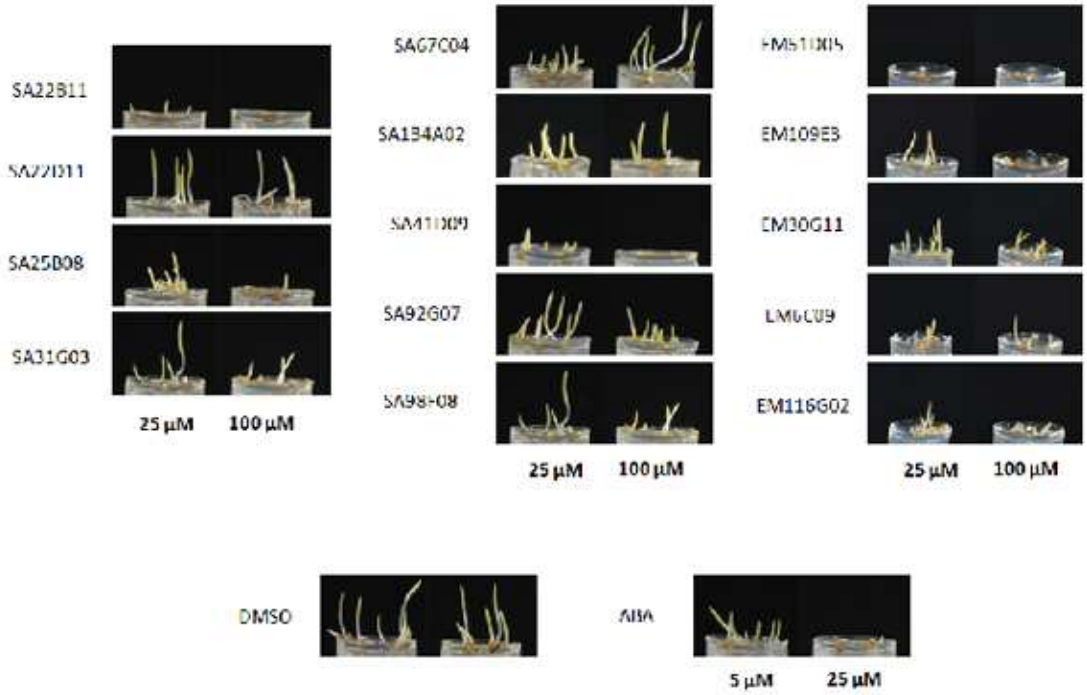

Figure 1.2. Partial or complete inhibition of germination in *P. virgatum*. Seeds were stratified for 3 days at 4 °C on agar media containing different concentrations of ABA or sulfonamide compound. Germination was scored 72 hours after stratification. The experiment was conducted in triplicate. Representative images for DMSO, 5 µM ABA, 25 µM ABA, and sulfonamide candidates at 25 µM and 100 µM.

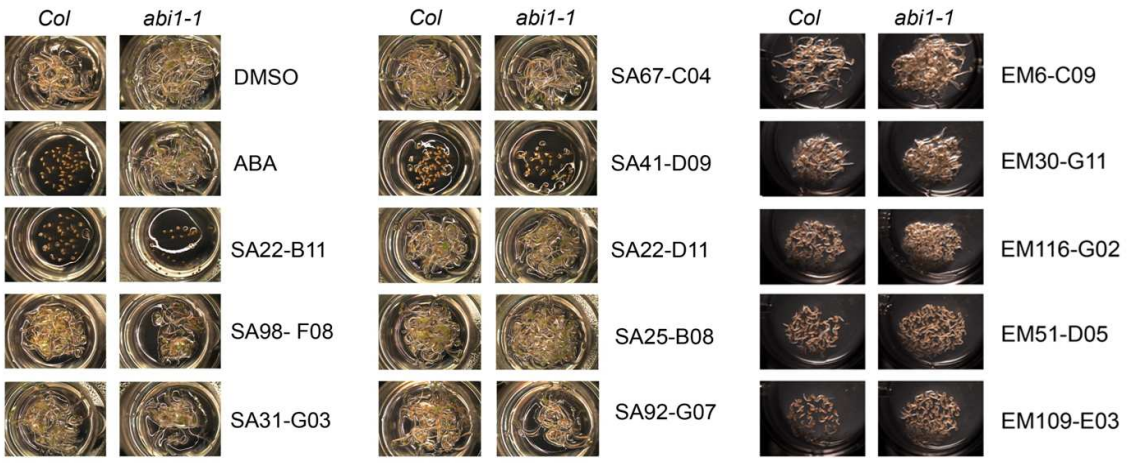

Figure 1.3. Germination sensitivity of sulfonamide candidates in *Arabidopsis thaliana*. Seeds of wild-type Columbia (*Col*) and ABA insensitive mutant (*abi1-1*) were monitored in the presence of 1µM ABA and 25µM if sulfonamide compound. The experiment was done in duplicate. Representative images for germination sensitivity in *Arabidopsis thaliana.*

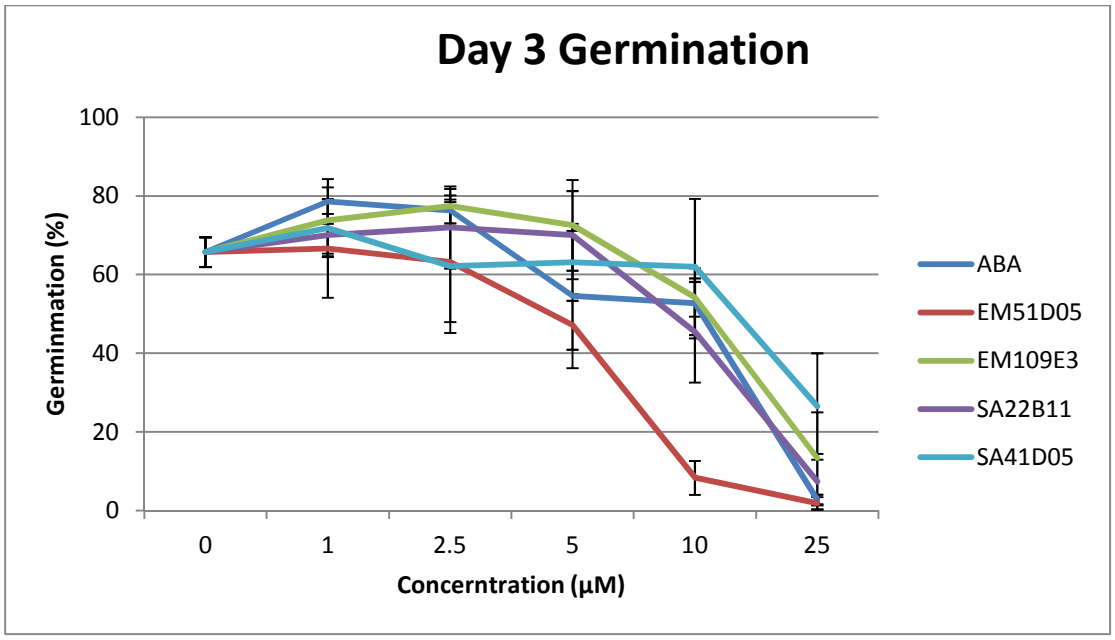

Figure 1.4. Germination inhibit of *P. virgatum*. Seeds on agar media containing different concentrations of ABA or sulfonamide candidate, and were scored 3 days follow stratification for 3 days at 4 °C. The experiment was conducted in triplicate. Values plotted are the average of three independent measurements. Error bars show standard deviation (SD).

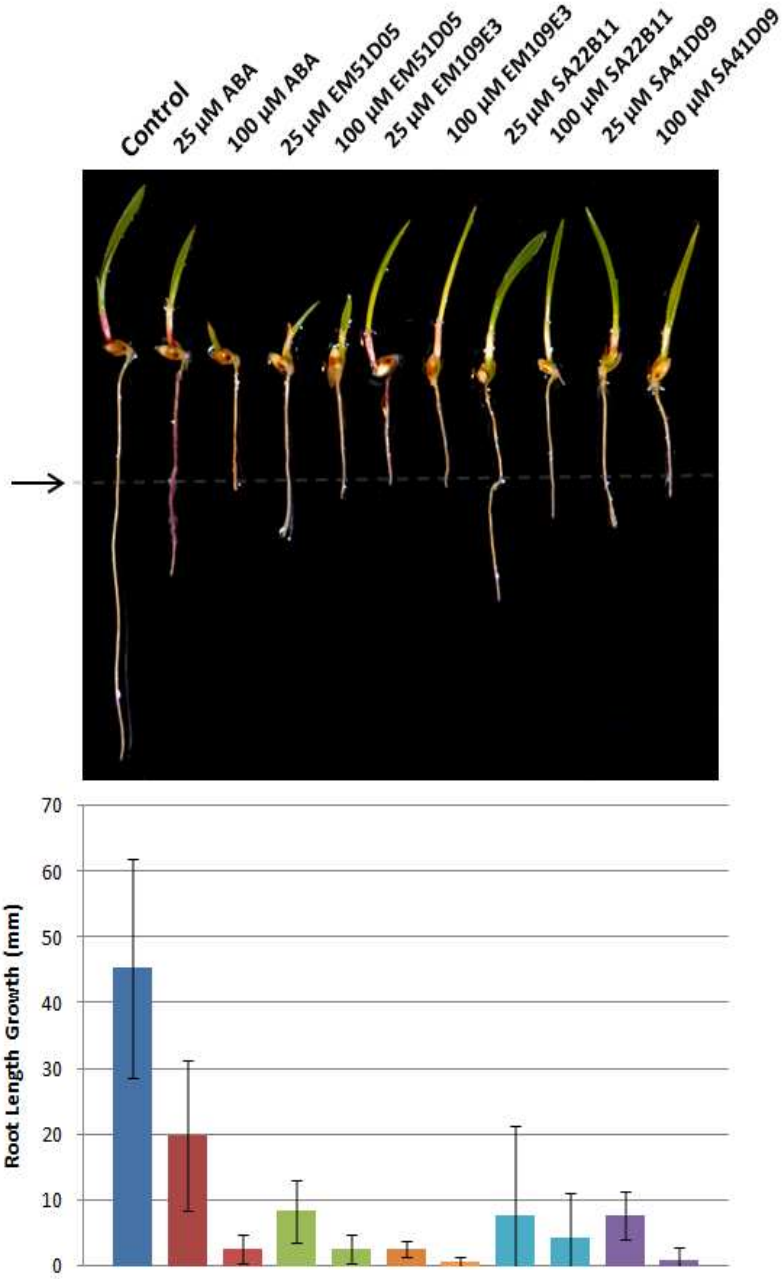

Figure 1.5. Root phenotype suppression in *P. virgatum* seedlings in the presence of potential ABA agonists. Seeds were stratified for 3 days at 4 °C on agar, allowed to germinate and grow for 4 days, before being transferred to agar media containing different concentrations of DMSO, ABA, or sulfonamide candidate. Seedlings were scored 3 days after using ImageJ. The experiment was conducted in triplicate. Representative images at 7 days post stratification for DMSO, 25 µM ABA or sulfonamide candidate, or 100 µM ABA or sulfonamide compound. Values plotted are the average of the three independent measurements and error bars show standard deviation (SD).

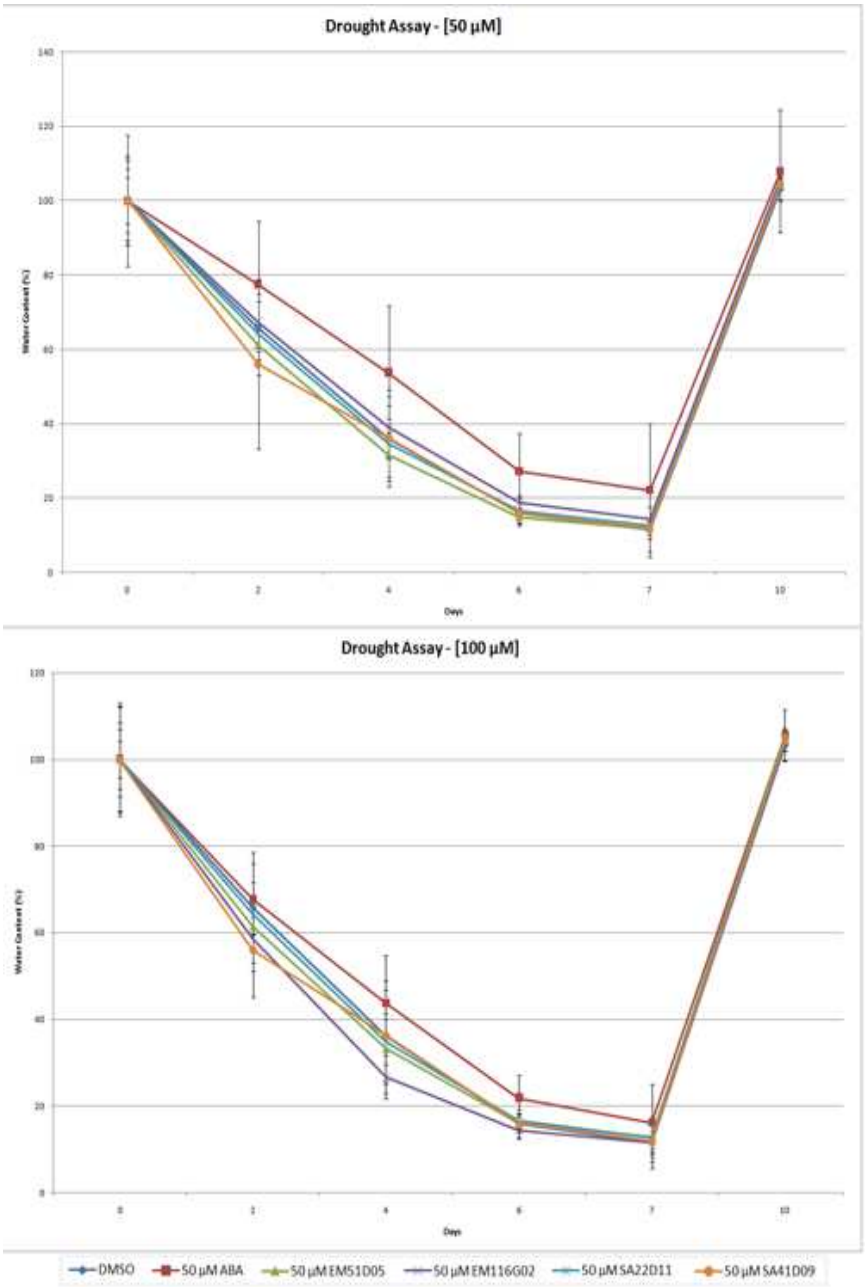

Figure 1.6. Drought stress assay on mature *P. virgatum* plants. Plants were sprayed with each respective compound at 50 µM on Days 0, 2, and 4. Water was limited until Day 7. Fitness and recovery were measured on Day 10 after rewatering. The experiment was conducted in triplicate, with 9 seedlings per replicate. Values plotted are the average of the three independent measurements and error bars show standard deviation (SD).

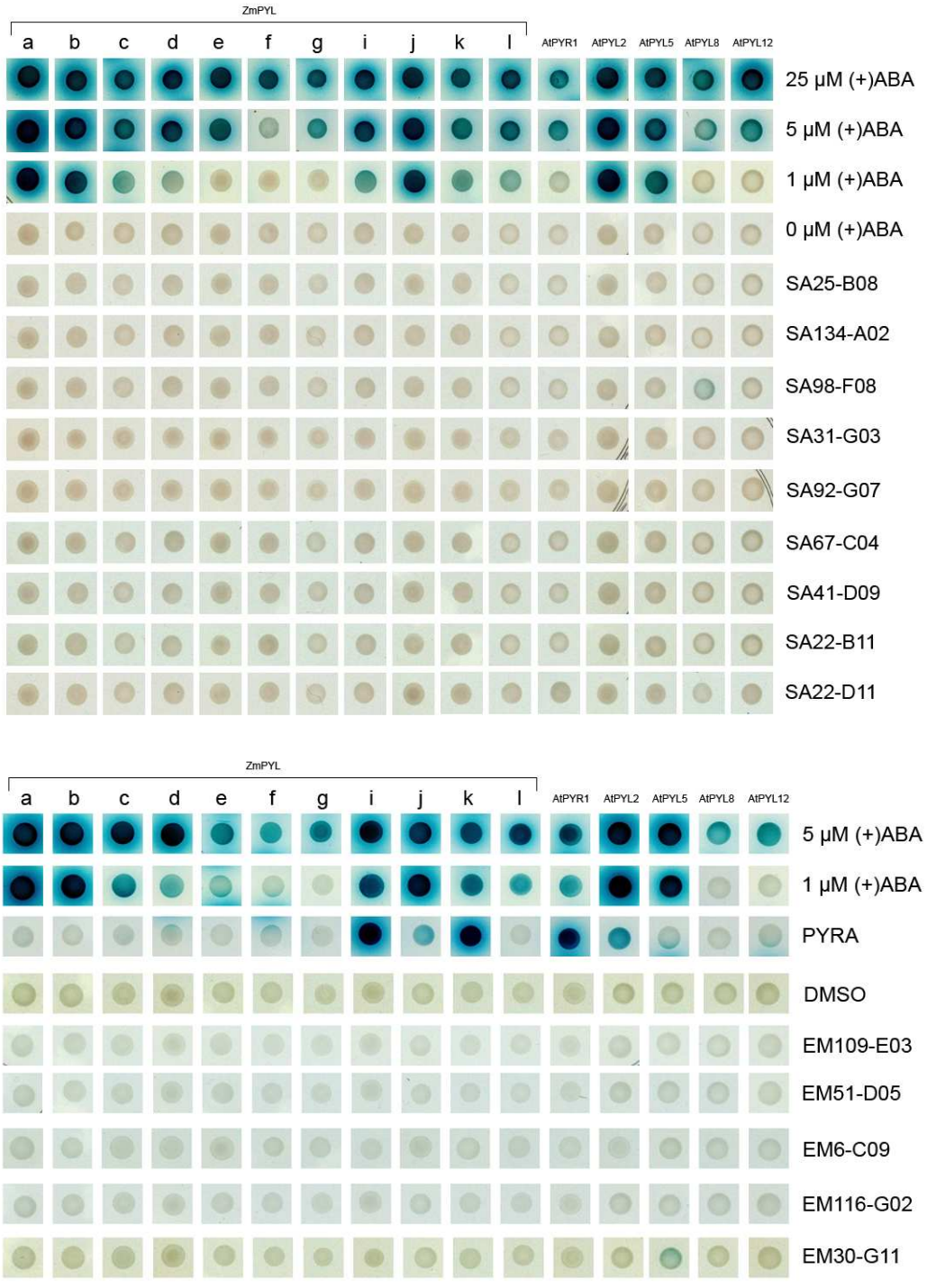

Figure 1.7. Y2H assay on *Z. mayes* and *A. thaliana* ABA receptors. All *Z. mayes* ABA receptors were screened for activity in the presence of ABA, Pyrabactin, or sulfonamide compound. Representative monomeric and dimeric receptors from *A. thaliana* were screened for activity in the presence of ABA, Pyrabactin, or sulfonamide compound.

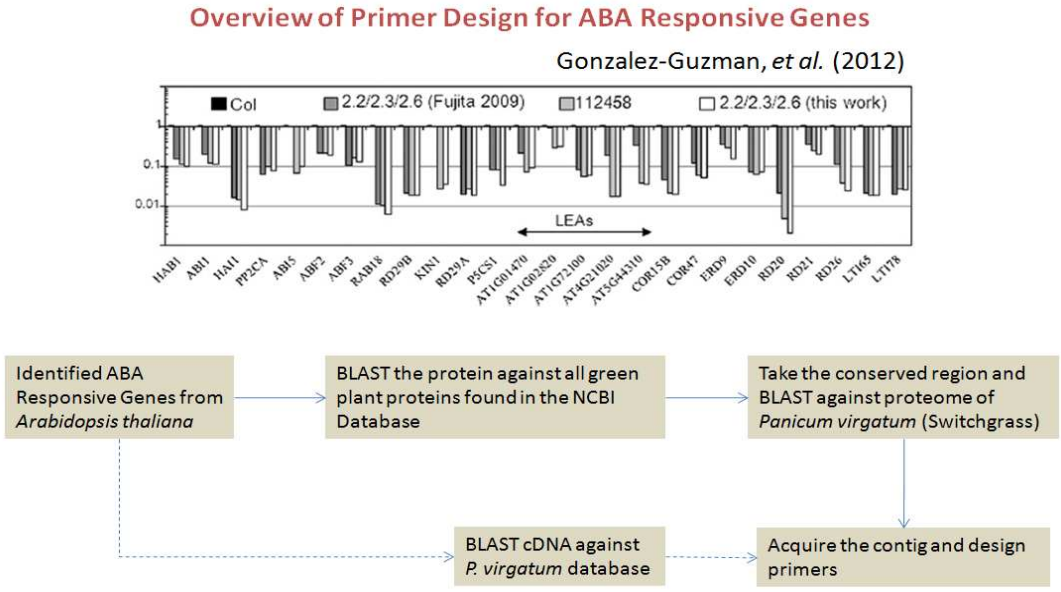

Figure 1.8. Overview of primer design for ABA responsive genes in *P. virgatum*. Genes were selected from work conducted by Gonzales-Guzman, *et al.* 2012. Protein sequences were BLAST screened from the NCBI database and conserved sequences were BLAST screened again the *P. virgatum* proteome. Primer design was done using the Roche Universal ProbeLibrary Assay **Design Center (Roche Applied Science).**

Gene	ABA Fold
PvHAB1	1.909
PvABF2	**4.648**
PvABF2(2)	3.863
PvABF3	3.299
PvABI1	3.966
PvABI5	1.081
PvABI5(2)	x
PvAT1G01470	2.777
PvAT1G01470(2)	x
PvABI1(2)	**5.245**
PvAT1G72100	x
PvAT4G21020	0.059
PvCOR47	**1166.048**
PvERD9	1.385
PvERD10	**8.535**
PvP5CS1	1.228
PvP5CS1(2)	**4.337**
PvPP2CA	3.723
PvRAB18	0.7423
PvRD20	1.176
PvRD21	0.411
PvRD26	3.846
PvHAI1	**4.16**
PvHAI1(2)	**4.713**
PvHAI1(3)	x
PvKIN1	0.955
PvKIN1(2)	x

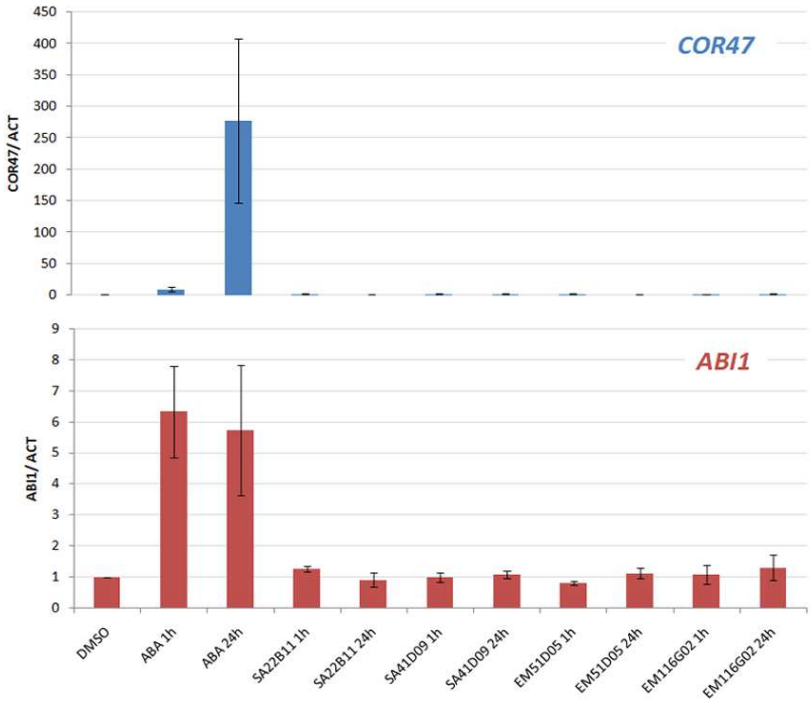

Figure 1.9. (Left) Induction of genes in the presence of ABA. Genes showing induction values greater than 4 are highlighted. (Right) Expression of ABA responsive genes 1 hour and 24 hours after chemical treatments. Actin (*ACT*) was used as control. The experiment was conducted in triplicate. Values plotted are averages of each individual replicate. Error bars show standard deviation (SD).

Primer Name	Forward Primer	Reverse Primer
qPvHAB1	ggcgaaggatgatgaatgtc	gttcgacattacgtcccagag
qPvABF2	aagcctcctgctatggagaa	tcccggttcttgatcatcc
qPvABF2(2)	cctggaagtggtcagcaatc	ccagggacctgtcaatgttc
qPvABF3	cctggaagtggtcagcaatc	ccagggacctgtcaatgttc
qPvABI1	ggcgaaggatgatgaatgtc	gttcgacattacgtcccagag
qPvABI1(2)	agcggttgtcgtggtagtgt	aacccgtgaatctccacagt
qPvABI5	aggccgaattgaaccatct	ttgtattctcctcggctttga
qPvABI5(2)	tccatcatgtcgctgacg	tcatggacccgaagttgc
qPvAT1G01470	ccatcatcggaaacttcacc	gagggttgggagcttgaact
qPvAT1G01470(2)	tcgccttcaagaacgtcag	tgcgagtaggggttgttga
qPvAT1G72100	cgccaaggacaaggtcac	cgatctttcgcgctcttg
qPvAT4G21020	cgccaaggacaaggtcac	cgatctttcgcgctcttg
qPvCOR47	cagctccagctcgtctgag	cttctccttgaggcccttct
qPvERD9	agcttcaaaggcctgaggta	ctcgctcttgtttccgaaga
qPvERD10	gggcacaaggacaaccag	cgacccagcataccctgt
qPvP5CS1	gtgatggatgccaaaattga	aacaagtaatgtttccatggcatt
qPvP5CS1	ttcaagatggttgaagaagttgtc	gcatcagcaatatccagcaa
qPvPP2CA	atttcttcggcgtctttgac	atcctgtcctggcacaacc
qPvRAB18	cgccaagcctactagctgat	cttccggtggcttctgttt
qPvRD20	gctcccagtatcgttcttcg	tccctgctgagtgggaaa
qPvRD21	gaccaatggagggaacagtg	atgaactcaaaggcggaatc
qPvRD26	tggctacttgcagtcgatga	tggtggcaagattgtctgc

Table 1. List of primers designed from the 19 ABA responsive genes characterized in *Panicum virgatum*. Primer design was done using the Roche Universal ProbeLibrary Assay Design Center (Roche Applied Science).

CHAPTER 2

ABSTRACT

Clade A subfamily of type II C protein phosphatases (PP2C) is negatively regulated by a family of star-related lipid-transfer (START) proteins called PYR/PYLs (pyrabactin resistance1/PYR1-like). *In vivo*, these proteins are activated when bound to Abscisic acid. This interaction stabilizes its conformation, leading to ABA signaling. Previous work done by Mosquna, *et al.*2011 were able to use that mimicked the agonist-bound conformation leading to the activations of signaling *in vivo*. Multiple yeast two-hybrid screens were performed to construct PYL2 receptors that inhibited PP2Cs in the absence of ABA. Here we compare the efficiencies of the constitutive active PYL2 receptors under drought stress in comparison to wildtype. Receptors were tested using the drought inducible promoter Rd29A. Our results reveal that conditional activation of constitutively active PYL2 is sufficient to elicit drought stress tolerance.

INTRODUCTION

The phytohormone abscisic acid (ABA) is involved in a number of important biological processes in plants including development and response to environmental stress (Finkelstein, 2002; Nambara, 2005). *In vivo*, ABA binds to a family of star-related lipid-transfer (START) proteins called PYR/PYLs (pyrabactin resistance1/PYR1-like) that are essential for ABA signaling. In their active ABA-bound state, PYR/PYL proteins inhibit members of the clade A subfamily of type II C protein phosphatases (PP2Cs). This inhibition leads to the activation of sucrose non-fermenting-1(SNF1)-related protein

kinase 2 (SnRK2) kinases, leading to downstream phosphorylation of targets that modulate ABA responses. In the absence of ABA, the PP2Cs dephosphorylate the SnRK2 kinase and therefore repress ABA responses.

Intensive research around the mechanism behind ABA and its mechanism of action has been ongoing for decades since it was first identified in the 1960s (Park, *et al*. 2009). Due to the genetic redundancy of the PYR/PYL receptors, initial efforts towards genetic identification using approaches such as loss-of-function and gain-of-function mutations did not identify ABA receptor proteins. One drawback of such approaches is that most mutations are not conditional and this can limit the characterization of essential genes (Stockwell, 2000). The use of chemical genetics can, in principle, bypass this limitation because chemical inhibitors and activators can be used to conditionally alter the function of gene products. In general, this approach entails assembling mutation equivalents in the form of ligands, screening these ligands for bioactivity, and identifying the targets of these bioactive molecules (Stockwell, 2000; Das, *et al*. 2011). This method was one of the key tools used for elucidating the receptors involved in the ABA pathway. Prior to employing this method, biochemical and cell biological approaches suggested the existence of ABA binding proteins but it link these candidate proteins were never convincingly connected to the ABA response pathway (Finkelstein, *et al*. 2002). In 2007, a selective ABA agonist named Pyrabactin was isolated from a high-throughput chemical screen (Zhao, *et al.* 2007). Genetic analyses identified a START protein calledPYR1 (pyrabactin resistance 1) as the cellular target of pyrabactin and the founding 13-member of family Arabidopsis ABA receptors (Park, *et al*.2009).

Since their initial discovery, focus has shifted towards the characterization of individual receptor function. Towards this goal, constitutively active receptor mutants for various receptors have been constructed and used to reveal that the activation of a single receptor (PYL2) is sufficient to elicit a multiple ABA responses in Arabidopsis (Mosquna, *et al.*2011). To further investigate receptor function using constitutively active receptors, we monitored the effects of the constitutive expression of PYL2 in *Arabidopsis* plants during abiotic stress and development to further dissect its role in ABA signaling. RD29A-(a stress-inducible promoter) driven constitutively active transgenic plants were subjected to abiotic stress to evaluate their performance in comparison to wildtype plants. During simulated drought stress, constitutively active transgenic plants demonstrated increased drought tolerance than wildtype and Col-0. Similar findings were observed in plants subjected to NaCl stress. Our results show that the conditional activation of constitutively active PYL2 is sufficient to elicit drought stress tolerance.

RESULTS

Arabidopsis thaliana transgenic lines used for this assay were selected after screening for mutants that conferred glufosinate herbicide resistance. After lines with single copy insertions were isolated, homozygous lines were also screened in the similar manner. After selecting homozygous representatives for transgenic lines expressing the Rd29A-driven GFP-tagged constructs in *PYL2* and *PYL2CA4*, we focused our efforts on characterizing the role of this receptor during abiotic stress. We first examined the

response of whole plants from Rd29:GFP-PYL2 and Rd29:GFP-PYL2 CA4 transgenic lines in regards to water use efficacy. Six week old plants were individually grown in Jiffy soil pellets and wrapped in polyvinyl-chloride and Parafilm plastic to simulate drought stress for a period of five weeks. In Rd29:GFP-PYL2 CA4, transgenic lines #17 and #21 were selected as representatives due to their desired expression patterns observed from preliminary assays. At the end of the week five, Rd29:GFP-PYL2 CA4 #17 and Rd29:GFP-PYL2 CA4 #21 transgenic lines retained more water on average (about 57% and 61%) compared to Col-0 and Rd29:GFP-PYL2 (about 41% and 51% respectively) as seen in Figure 2.1A. Plant weight was measured weekly throughout the experiment. No significant changes in plant weight were appreciated between the fourth and fifth week of drought simulation. From observation, a majority of water lost through transpiration occurred during the first and second week. Notable differences in phenotype, in particular plant weight and leaf turgor, were also observed during this time period and correlate to the data collected for average water retention. Leaf turgor is used as our quantifying factor which at week two demonstrated that the transgenic mutant lines, especially Rd29:GFP-PYL2 CA4 #17 and Rd29:GFP-PYL2 CA4 #21increased survivability. In comparison to untreated plants that are the same age, Rd29:GFP-PYL2 CA4 water deprived plants look fairly similar in appearance and size (Figure 2.1B).

In attempt to explain the phenomena appreciated from our drought assay, we quantified stomatal aperture under experimental conditions. Stomatal aperture measurements through scanning electron microscopy (SEM) were conducted following induction and rehydration of leaf tissue from each plant line. Tissue from transgenic lines was sampled

and impression molded using the *Suzuki's Universal Micro-Printing (SUMP)* method. Our results show that Rd29:GFP-PYL2 CA4 #21 possessed smaller widths on average than Col-0 and Rd29:GFP-PYL2 CA4, suggesting that most of its stomata are closed during drought stress. Rd29:GFP-PYL2 CA4 #17 also produced smaller widths but not as significantly as Rd29:GFP-PYL2 CA4 #21. Consistent with the findings from the drought stress assay, closed stomata in both Rd29:GFP-PYL2 CA4 #17 and Rd29:GFP-PYL2 CA4 #21 suggest a reduction in transpiration leading to less water used by the plants. In contrast, Col-0 and Rd29:GFP-PYL2 CA4 had wider stomata widths on average, allowing more water to be lost through transpiration. Constitutive activation of PYL2 receptors produced stronger ABA response to drought stress in comparison to wildtype receptors.

We next examined the role of PYL2 in response to other forms of abiotic stress, in this case NaCl stress (Figure 2.2). In our NaCl resistance assay, one week old seedlings were acclimated in 100mM NaCl for 16 hours before being transferred to 250mM for two weeks. Survived seedlings was used as the quantifying factor in measuring NaCl resistance. Our findings show that PYL2 produced similar NaCl resistance as PYR1. Both PYR1 and PYL2 had similar tolerance to NaCl as Col-0. Overexpression of both PYR1 and PYL2 produced substantially resistance to NaCl in comparison to wildtype and Col-0.

DISCUSSION

Previous work from Mosquna *et al.* 2011 demonstrated the ability of PYL2 to sufficiently activate ABA signaling in vivo even when expressed in mutants that are deficient in ABA-mediated seed response. They were able to selectively activate individual family members of ABA receptors but have not explored the possibility of each having different subfunctions, since ABA activates multiple receptors in wild-type plants.

In this report, we continue this phenotypic exploration by characterizing the function of constitutive active PYL2 during abiotic stress such as drought and salt exposure. We tested this construct using the stress-inducible promoter Rd29A. Our data demonstrates that expression during both drought and salt stress in PYL2 is sufficient to activate ABA response in vegetative tissue. A stronger response was observed in overexpressed transgenic plants. One caveat of expression of the PYL2 construct in null mutant background is that rosette size is reduced. This phenotype is seen in both treated and non-treated plants of the same age during our drought assay. Overexpressed CA4 lines of the PYL2 construct shows a more drastic reduction in rosette size at day 7 in comparison to both Col-0 and Rd29:GFP-PYL2. This size differential is also prominent in control plants at day 14. At day 14 of water deprivation, there are minor differences in size compared to both Col-0 and PYL2. Drought sensitivity decreases in CA4 mutants. This assay will need to be repeated using developmentally equivalent plant material. The difference in size can be a contributing factor to average water retained at the end of the drought simulation. The reduced surface area of CA4 transgenic lines could result in less

transpiration compared to Col-0 and PYL2. However, at day 14 of the drought experiment this size difference is indistinguishable in Col-0, Rd29:GFP-PYL2, and Rd29:GFP-PYL2 CA4 lines. We were able to address this theory by measuring stomatal aperture in attempts to find linkage to the observed phenotypes. Consistent with our drought assay findings, Rd29:GFP-PYL2 lines had more stomata closed on average than Col-0 and Rd29:GFP-PYL2. Constitutive activity produces a baseline biological response in the absence of a bound ligand. One outcome of ABA signal activation is the closing of stomata. This baseline response results in a decrease in transpiration. In our NaCl resistance assay, overexpression of the CA4 receptors produced tolerant vegetative plants when exposed to 250mM NaCl. This decrease in sensitivity was seen in both Rd29:GFP-PYR1 and Rd29:GFP-PYL2 CA4. Rd29:GFP-PYR1 and Rd29:GFP-PYL2 wild-type lines demonstrated similar NaCl sensitivity as Col-0. Collectively, our data demonstrates that the expression of Rd29:GFP-PYL2 CA4 is sufficient to activate signaling in vegetative tissue. Stress tolerance is noted in constitutively active transgenic lines is based on fitness. Together with previous data, we continue to gain more insight on the individual functions of each receptor involved in ABA signaling. Future work is expected towards characterizing the functions of the remaining receptors using similar assays in hopes to gain a complete picture of their roles in ABA signaling.

MATERIALS AND METHODS

Plant material and growth

Transgenic *A. thaliana* lines: Col-0, Rd29:GFP-PYL2 #19, Rd29:GFP-PYL2 CA4 #17,and Rd29:GFP-PYL2 CA4 #21, were selected for single copy T-DNA inserts using glufosinate resistance segregation. Glufosinate resistance was also used to identify homozygous transgenic lines. Transgenic seeds were surface sterilized in Ependorff tubes using the following solutions: 70% EtOH for 5 minutes, 39.5ml Water, 10ml 5.25% (w/v) hypochlorite bleach, 500uL 10% Tween-20 for 5 minutes, and washed 5 times with water. Seeds sewn onto 1/2 Murashige and Skoog Basal *Salt* Mixture (MS), 0.5% Sucrose, and 0.7% Agar containing glufosinate using 0.1% Liquid Agar, stratified for 5 days in dark at 4°C, and grown in 16h light, 8h dark growth chambers at room temperature (25°C). Transgenic lines that did not segregate glufosinate sensitivity, yellowing of the cotyledon, in the T3 seed, but were segregating 3:1 in the T2 seed were classified as homozygous. After isolating single copy and homozygous representatives for each genotype, seeds were grown and sewn onto petri dishes containing 1/2 (MS), 0.5% Sucrose, and 0.7% Agar containing 100mg/ml Carbenicillin antimicrobial agent. Sewn seeds were stratified at 4°C in the dark for four days to and grown 16h light, 8h dark at room temperature for five days. For drought assays, seedlings were transferred to Jiffy-7 peat pellet soil and grown for 6 weeks.

NaCl Resistance assay

Homozygous seeds were sterilized, sewn, and germinated onto gridded plates containing in BD Falcon 100x15mm Disposable Square Integrid Petri Dishes on media consisting of 1/2 x Murashige and Skoog Basal *Salt* Mixture (MS), 0.5% Sucrose, and 0.5% Gelzan™ Agar, and 100mg/ml Carbenicillin antimicrobial agent. Following media sterilization, a sterile 80x80mm Nylon Mesh (Mesh Cat#: B001UTS000) was placed on top of 25ml liquid media prior to solidification. A1000 microns square openings nylon mesh used have with 59% open area, and 515 micron thread diameter was utilized. Nine seeds were sown evenly on each plate over 10x10 mesh square units. Sown plates were sealed with 3M Micropore tape and stratified in darkness at 4°C for 5 days. After 5 days stratification, seeds were grown in chambers set at 25°C, 16 h light, 8 h dark. After 1 week, seedlings were transferred to plates containing similar mesh media plates in addition to 100uM NaCl. The transfer was done by transplanting the mesh containing the seedlings onto the mesh media plate containing 100mM NaCl. Seedlings were acclimated for sixteen hours in chambers set at 25°C, 16 h light, 8 h dark. An additional mesh layer was placed on top of solid media before transfer to prevent the seedlings from making contact with the NaCl surface. Plates were then sealed with 3M Micropore tape. Acclimatized seedlings are then transferred onto mesh media plates containing 250uM NaCl and sealed with Micropore tape. A strip of Parafilm is placed on three of the four edges of the plate to allow a small window for ventilation. Survival rates were scored after 14 days or until 60% of Col-0 has survived.

Drought assay

Seedlings were individually transferred to hydrated Jiffy-7 peat pellet soil and placed in an individual 2.25 inch by 1.33 inch Terracotta pot (Farrand Enterprises). Forty plants per genotype were grown for 6 weeks. Mature plants were prepared for drought simulation by using a combination of polyvinyl-chloride and Parafilm plastic to limit any factors of water loss not attributed to transpiration. The outer surface of the pot is covered in polyvinyl-chloride and placed into an empty pot. The upper half of the apparatus wrapped with Parafilm plastic, only exposing the shoot and biomass of the plant. Five plants per genotype, used as biological controls, are grown in parallel to treated plants. Plants were then imaged and quantified for total weight. Data for desired parameters were collected every week, one week following prior measurements for a period of 3-5 weeks. After this measurement period, individual plants were dissected and part of the apparatus was taken apart to determine plant dry weight and relative water content. Plant biomass and soil were dried in an oven for a week. All parameters collected were then processed to determine the plant water content and water lost by the plants through transpiration.

Stomata Assays

Col-0, Rd29:GFP-PYL2 #19, Rd29:GFP-PYL2 CA4 #17,and Rd29:GFP-PYL2 CA4 #21 seed were surface sterilized, sewn, and stratified in dark at 4°C for 5 days. Seeds were sown side by side across a OmniTray Polystyrene 128 x 86 mm lawn plate on media containing1/2 x MS, 0.5% Sucrose, and 0.7% Agar. After stratification, plates are transferred to growth chambers at 23°C on a 16h light, 8h dark cycle and grown vertically

for 1 week before being transferred to soil. After 4 weeks, leaf tissue was taken from each plant for stomatal measurements. Leaf tissue was dried under light in a sterile hood for 1h (induction time). Plants were reintroduced to water for 1h 30m. Following induction treatment, stomatal morphology was captured by impression molding using the *Suzuki Universal Micro-Printing* (*SUMP*) method (*SUMP* Laboratory, Tokyo). Each measurement was done in triplicate. Prior to each mold, *SUMP plates* were incubated by exposing the disks to 20uL of *SUMP liquid* (isopentylacetate).This allows the plastic to become malleable for a limited period of time. Leaf tissue is removed after incubated disks have solidified. Each transgenic line was sampled in triplicate. The impressions were imaged using a TM1000 Hitachi Tabletop SEM at X1200 magnification. Around 100 stomata per transgenic line were captured for each transgenic line. Width and lengths measurements were obtained using ImageJ 1.45 (Rasband W. National Institutes of Health, USA). and quantified.

Western Blot Analyses

One equal-sized rosette leaf from 4 week old transgenic plants was obtained for western analysis, frozen in liquid nitrogen, and pulverized in tubes containing one 6.35mm diameter chrome steel ball and two 3.2mm diameter chrome steel balls (BioSpec Products)**.** 10µL TBS buffer [10mM Tris·HCl (pH 7.4) 150 mM NaCl] supplemented with 1% protease inhibitor mixture (Sigma) was added to ground tissue, which was then

allowed to thaw and mixed thoroughly. Samples were then centrifuged for 10 minutes at 4^{o}C. 30µL of each sample is added to a PCR tube containing 30µL of sample buffer. 20µL of protein was analyzed by SDS-PAGE with10% acrylamide gel (wt/vol). Immediately load stacking gel mixture into casting module with a pipette and insert gel combs. After separation, the separated proteins were transferred on to nitrocellulose membranes using semi-dry transfer and stained by Ponceau S, Acid Red 112 for rapid detection of protein bands. After dye removal by washing, the membranes were blocked overnight in 5% Milk TBST. The blocked membranes were probed with either an anti-GFP (Clontech) or anti–α-Tubulin (Sigma) antibody at 1:10,000 dilution for 1 hour then washed 3 times in 0.1% TBST, each wash being 10 minutes. The membranes were then probed for 1 hour using an anti-mouse–HRP (1:10,000) conjugate as the secondary antibody. Signal was detected using ECL (GE Healthcare).

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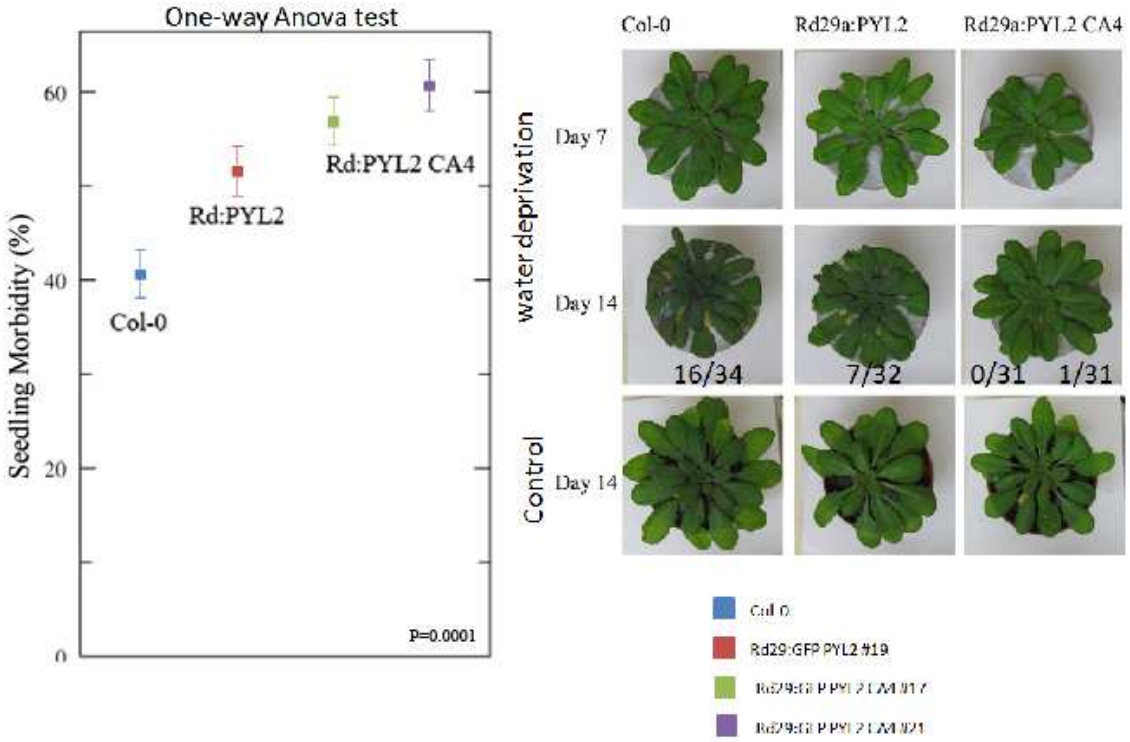

Figure 2.1. Seed Morbidity of *Arabidopsis thaliana* in response to drought stress. Six week old plants were subjected to five week drought simulation. (A, Left) Values plotted using One-way Anova test with a P-value of 0.0001. (B, Right) Representative images of plant morbidity at Day 7 and Day 14 under water deprivation and Day 14 under control treatment. Leaf turgor was scored at Day 14 under water deprivation.

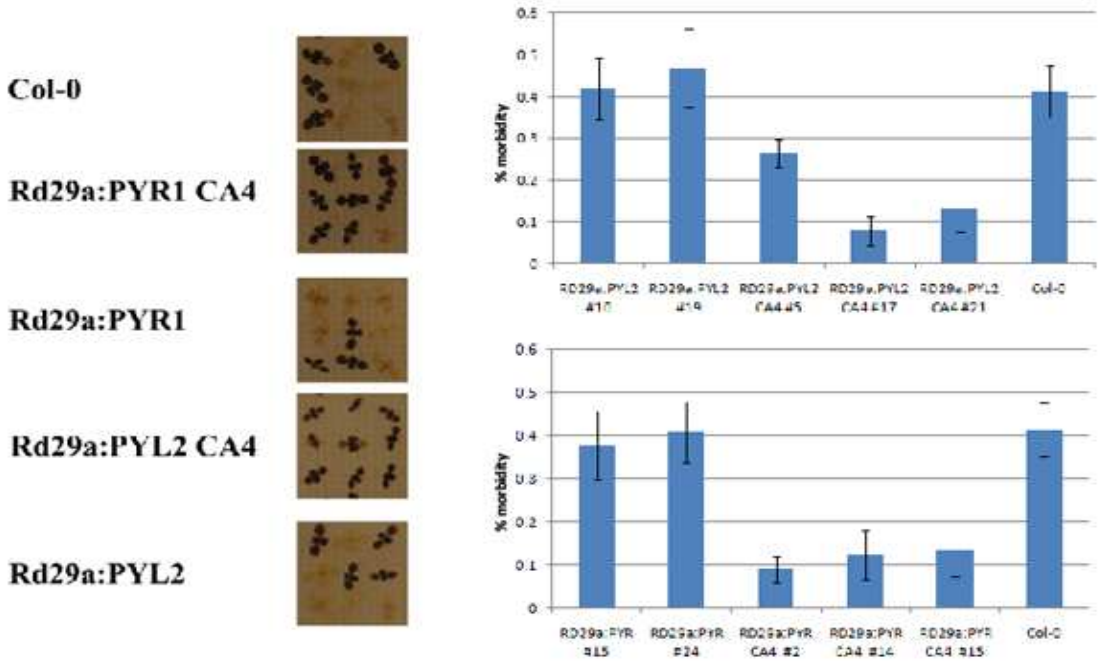

Figure 2.2. NaCl resistance assay on one week old seedlings. Seedlings were acclimated 100mM NaCl for 16 hours before being transferred to 250mM NaCl for two weeks. (Left) Representative images after two weeks after 250mM NaCl exposure for wildtype and transgenic lines. (Right) Average percentage of plant death after two week exposure to 250mM NaCl in wildtype and transgenic lines. Values plotted are the average of three independent measurements. Error bars show standard deviation (SD).