Understanding the Abscisic Acid Pathway Using Guard Cell Specific Genes and the Anti–Aging Drug Spermidine

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Abstract

Plants must respond to environmental stress including drought and harsh winters. Overcoming these stresses depend heavily on timing of stomatal closure and seed germination. My research focused on both chemical and genetic aspects involved in the abscisic acid pathway that controls both stomatal closures in leaves and seed germination in *Arabidopsis thaliana*. My first study focused on recognizing specific proteins involved in the abscisic acid pathway for stomatal guard cell closure. Given specific promoters for their respective proteins, it is possible to determine whether or not proteins are guard cell specific. Gateway™ technology utilizes a series of reactions to create a clone containing a promoter of interest called an expression vector. By injecting this vector directly into the leaf of *Arabidopsis,* the plant will use the promoter to create the guard cell specific protein. The second study examined the effect of the anti-aging drug Spermidine on seed sensitivity to abscisic acid concentration during seed germination. By varying the concentration of Spermidine and abscisic acid exposure to seeds then observing the number of surviving seeds, the effects of Spermidine on seed germination can be measured. Spermidine is expected to reduce seed sensitivity to abscisic acid leading to increased seed germination.

Introduction

Plants must respond to environmental stresses such as droughts and harsh winters. Each year billions of dollars are lost due to drought and winter. The National Weather Service reports that drought alone cost the U.S. over $61.6 billion. Making plants that are better suited for combating these disasters has become a major area of concern. There are ways to do just that. Overcoming these stresses depend heavily on timing of stomatal closure and seed germination.

Stomata are microscopic pores on the bottom surface of leaves. Two guard cells make up the pores which control gas exchange for the plant during photosynthesis (Pei et al., 1998; Hugouvieux et al., 2001). As gases are being exchanged water escapes. This becomes a great problem for plants during drought. Opening stomatal pores contribute to 95 percent of total water lost from the plant. By genetically controlling when stomata open and close, a plant can conserve the precious water it needs to survive when water becomes scarce.

The anti-aging drug Spermidine controls many cellular processes to slow the aging process down in organisms. In plants seed germination is slowed keeping the seeds alive during the winter. This is critical for long, harsh winters during which, seeds die before the rains and rising temperatures permit optimal growth. By controlling when a seed germinates, a seed can endure the winter long enough for those favorable conditions to arrive in the spring. Greater understanding of these processes will give plants necessary tools for coping with intense weather conditions.

Common to these seemingly unrelated events are their signaling mechanisms, the abscisic acid (ABA) pathway. My research focused on both chemical and genetic aspects involved in the ABA pathway. Despite ABA's role in stomatal movements, genetic components of the humidity signaling cascade remain largely unknown. Thus, my first study focused on the genetic aspect by identifying regulatory regions in guard cell preferential genes. These genes are responsible for stomatal guard cell closure involved in the ABA pathway. Controlling when the gene is expressed means finding the promoter. So developing a faster method to identify the region in the promoter specifically that encodes functionality is of the greatest importance. Guard cells use a complex signaling network to create a "graded binary" output that can readily be observed under the microscope: stomatal 'opening' or 'closing'. The study of guard cell signaling provides insights into how the many cellular processes assemble together to create a quantifiable single

cell output (Kwak et al., 2008), allowing quantitative dissection of the functions of individual genes and proteins within signaling cascades.

Despite its role in stomatal movements, molecular components of the humidity signaling cascade also remain largely unknown. Humidity is an environmental stimulus that regulates stomatal movements. Stomatal closure occurs very rapidly in response to a reduction in relative humidity in the atmosphere. So when the air has very little moisture, stomata close.

Genomic techniques have been developed and adapted to *Arabidopsis thaliana* guard cell signal transduction studies in humidity. This allows for molecular genetic, cell biological, biophysical, physiological, and functional genomic analyses of single cell signaling responses (Pei et al., 1997; Pei et al., 1998; Allen et al.,1999; Wang et al., 2001; Hosy et al., 2003; Leonhardt et al., 2004) in *Arabidopsis*. *Arabidopsis* provides a model for understanding stomatal closure and seed germination. It is a small plant, allowing it to be used in vitro, meaning in controlled conditions. It has a short life cycle for quick growth. Thousands of seeds can be collected from a single plant. Its small genome was sequenced and annotated. Exchanging DNA between it and bacteria is relatively simple. This all contributes to the adundance of genetic and genomics tools available for plant experimentation. Recently, most biochemical, genetic and molecular studies on plant hormone action and biosynthesis have been carried out in the model plant *Arabidopsis thaliana* for its simplicity of genome, short reproductive cycle, small plant size and great amount of information available on its metabolic pathways and signaling (Tassoni, et al., 2000).

Being targets of early signaling branches, ion channels provide effective functional signaling and quantitative analysis points to identify and characterize upstream regulators and identify the intermediate targets and signaling branches that are affected either directly or indirectly by these regulators. Regulatory factors that control ABA response have been identified by genetic, biochemical, and pharmacological/cell biological approaches (reviewed by Rock, 2000; Finkelstein and Rock, 2002). As mentioned previously, identifying the region in the promoter specifically that encodes functionality is necessary. Biochemical approaches have uncovered a variety of gene promoter elements such as kinases, kinase inhibitors, phosphatases, phospholipases, and transcription factors that correlate with the ABA response (Finkelstein et al., 2002). Having identified a series of transcriptional activators and their target genes, it should be possible to dissect the signaling pathway involved in the ABA activation of these [promoter elements] (Finkelstein et al., 2002).

ABA mutant phenotypes may be tissue specific and subtle (Finkelstein et al., 2001). Given tissue specific promoters, it is possible to determine whether or not the genes activated are guard cell specific using Gateway™ technology. Gateway™ technology utilizes a series of reactions to create a clone containing a promoter of interest called an expression vector. By injecting this vector directly into the leaf of *Arabidopsis*, the plant will use the promoter to activate the promoter elements in guard cells specifically.

Recall that plants must respond to both drought and harsh winters. Surviving harsh winters depends on timing of seed germination. The second study examined the effect of Spermidine on seed sensitivity to ABA concentration during seed germination.

ABA is required for plant adaptation to environmental stress by affecting different plant tissues, developmental stages, and physiological processes. These include changes in seed dormancy and germination (Leung and Giraudat, 1998). Spd allows for inhibition of the phase transitions from embryonic to germinative growth and from vegetative to reproductive growth (Leung and Giraudat, 1998; Rock, 2000; Rohde et al., 2000b). Thus, seed germination is slowed keeping the seeds alive during the winter.

The study examined the effect of Spermidine on seed sensitivity to ABA concentration during seed germination. Although endogenous ABA [produced by the plant] is essential for the induction of dormancy and the germination of mature *Arabidopsis,* the seed can be suppressed by as little as 3 uM exogenous ABA (Finkelstein et al., 2001). Genetic studies in *Arabidopsis* demonstrated that the first major ABA accumulation phase is maternally derived and immediately precedes the maturation phase of seeds (Karssen et al., 1983). The second major ABA accumulation in wild-type *Arabidopsis* seed depends on synthesis in the embryo itself (Karssen et al., 1983). Although the embryonic ABA accumulates to only one-third the level accumulated at 10 days after pollination, it is essential for the induction of dormancy, which is maintained despite a substantial decrease in ABA by seed maturity (Finkelstein et al., 2001). The ABA content of a wild-type mature dry seed is only 1.4-fold that of the peak ABA level in a nondormant ABA-deficient mutant, suggesting that endogenous ABA is not the only signal for dormancy maintenance in mature seed. Despite the strong evidence for a fundamental role of ABA in regulating dormancy, this is a complex trait controlled by many factors (Finkelstein et al., 2001). Seed maturation begins when developing embryos cease cell division and

start growing and is correlated with an increase in seed ABA content, consistent with the fact that ABA can induce the expression of a cyclindependent kinase inhibitor (ICK1) (Wang et al., 1998) that would lead to cell cycle arrest at the G1/S transition (Finkelstein et al., 2000) stopping seed germination.

By varying the concentration of Spermidine and abscisic acid exposure to seeds then observing the number of surviving seeds, the effects of Spermidine on seed germination can be measured. Spermidine interacts with macromolecules like DNA, RNA, acid phospholipids and proteins (Tassoni et al., 2000). In plants, they have been implicated in a large range of growth and developmental processes including germination of seeds and response to environmental stresses (Buuren et al., 2000).

Based on the results of a similar study, the authors suggest that Spd may prevent chilling injury during harsh winters in squash by a mechanism involving protection of membrane lipids. Chilling injury is thought to involve alteration of membrane structure. Raison and Lyons proposed that the primary event causing chilling injury is a phase transition in the molecular ordering of membrane lipids. The membrane phase transition would have many deleterious effects on the tissues, including increases in membrane permeability and alteration of the activity of membrane proteins. All the results support the view that Spd and may inhibit chilling injury by slowing [lipid disfigurement] in Arabisopsis (Bouchereau et al., 1999).

Spermidine is expected to reduce seed sensitivity to abscisic acid leading to increased seed germination. At [0.5 mM] concentration, 100 % of seeds germinate (data not shown) to clearly demonstrate that several plants responded to low temperature acclimation with a uniform and substantial increases in Spd (Tassoni et al., 2000). An inhibition of the seed germination percentage was also observed [when too much Spd is added] (Bouchereau).

Methods for Determining Guard Cell Specific Genes

Stock and working solution preparation

The primers were obtained and prepared by adding necessary amounts of water. Afterwards, each solution was mixed using Vortex and spun using a bench top centrifuge. The working solutions were prepared by adding 5 uL of the primer solution to 45 uL of water into a 1.5 mL Eppendorf centrifuge tube. These solutions were also mixed and spun. They were then placed in ice.

Genomic DNA preparation

To prepare genomic DNA, 2 *Arabidopsis* leaves were cut and placed into 1.5 mL microcentrifuge tubes. This was repeated 20 times. Added to each tube was 100 uL of Edward's buffer. Using a pestle, the leaves were ground. The mixtures were centrifuged for 15 minutes at 14,000 rpms. The supernatant was transferred to new tubes. Then added to the solution was 450 uL of isopropanol to precipitate the mixture. It was inverted 5 to 10 times, then left to incubate at room temperature for 15 minutes. Afterwards, the mixtures were centrifuged for 15 minutes at 14,000 rpms. The precipitate was removed, resuspended, then centrifuged at 14,000 rpms for only 5 minutes. The DNA now could be resolubilized and separated by adding 100 uL of autoclaved water. Then the precipitate was resuspended using the pestle. The DNA was then incubated in the shaker for 15 minutes at 60°C. The DNA was resuspended without the pestle and centrifuged at 14,000 rpms for 15 minutes. The supernatant, now containing the DNA, was extracted and stored at –20°C.

Amplified promoters using PCR for GoTaq enzyme

First, the substances were thawed, mixed, and spun except for the enzymes obtained. The GoTaq solution was prepared as follows. It included 5 uL of buffer solution, 14 uL of autoclaved water, 1 uL of reverse and forward primers, 2 uL of dNTP, 1 uL of $MgCl_2$, 0.5 uL of gDNA and 0.5 uL of polymerase. PCR tubes were obtained and solutions were transferred to the tubes. The PCR was run using the following settings. First, 95°C for 2 minutes, 95°C for 20 seconds, 55°C for 20 seconds, 72°C where time depends on length of primer (1 minute per kb), 72°C for 5 minutes, and 4°C infinitely.

Agarose gel electrophoresis preparation

The gel was prepared by adding 30 mL of TAE per 0.3g of agarose into a 500 mL Erlenmeyer flask. The solution can only be dissolved by heat. Thus, the solution was placed into the microwave for 1 minute. The solution was then cooled by transferring it several times between a 250 mL Erlenmeyer flask. Then, 0.3 uL of Ethidium Bromide was

added to the smaller flask per 30mL of solution. The solution was swirled and then poured into the electrophoresis tray. It took approximately 15 minutes to polymerize.

Agarose gel electrophoresis

The GoTaq solutions were each placed into a well. Then, 6 uL of Ladders was added to the last well. It was used as a reference for size of DNA fragments. Then, the gel was run at 135 Volts until the fragments migrated to approximately two-thirds of the gel. Then using Quantity One: Gel Doc XR™ program, pictures were taken for length confirmation. If a positive match, then the promoters of interest were amplified using KOD. If not, the process was repeated to confirm the negative result from the GoTaq enzyme.

Amplified promoters using PCR for KOD enzyme

First, the substances were thawed, mixed, and spun except for the enzymes obtained. The KOD solution was prepared as follows. It included 5 uL of buffer solution, 32.5 uL of autoclaved water, 1.5 uL of reverse and forward primers, 5 uL of dNTP, 3 uL of $MgSO_4$, 0.5 uL of gDNA and 1 uL of polymerase. PCR tubes were obtained and solutions were transferred to the tubes. The PCR was run using the following settings. First, 95°C for 2 minutes, 95°C for 20 seconds, 55°C for 20 seconds, 70°C where time depends on length of primer (25 seconds per kb), 70°C for 5 minutes, and 4°C infinitely.

Agarose gel electrophoresis

The gel was prepared in the same way as previously described. Before being added to the wells, one-fifth of KOD's volume in 6X Sample Buffer dye was added to each PCR tube. There was excess amount of KOD solutions so each was divided into two electrophoresis wells. After adding the KOD solutions, 6 uL of Ladders was added to the final well. Then, the gel was run at 135 Volts until the fragments migrated to approximately two-thirds of the gel. Then using Quantity One: Gel Doc XR™ program, pictures were taken for length confirmation. If the lengths were correct, then the DNA would be extracted. If there was a negative result, the amount of GoTaq enzyme cycles was reduced to 10, purified, and then amplified using the KOD enzyme.

PCR purification

The PCR product was obtained and transferred to 1.5 mL microcentrifuge tubes. Added to the product was 62.5 uL of Binding solution I. This was then transferred to a separation column and centrifuged for 1 minute at 14,000 rpms. The flowthrough was discarded, then 500 uL of wash was added. The mixture was then centrifuged for 30 seconds at 14,000 rpms. The flowthrough was discarded and then the DNA was washed again. The residual wash was then removed via centrifugation for 1 minute at 14,000 rpms. The DNA was then eluted by adding 35 uL of water to the membrane, letting it incubate for 2 minutes, then centrifuging again for 1 minute at 14,000 rpms.

DNA Extraction

The promoter sequences were extracted from the gel using extraction pipette tips on an UV emitting table. It was important to expose the DNA for a very brief period of time due to sensitivity. Each piece of gel is equivalent to 45 uL. Using the Gel Extraction Kit, triple the volume of gel extracted is necessary to dissolve the gel. Thus, 270 uL of the Extraction Buffer was added to both 45 uL pieces of gel in a 1.5 mL Eppendorf tube. The extracted DNA was heated for 1 minute at 60°C to melt in the Thermo Mixer™. The mixture was transferred to the extraction column and centrifuged for 30s at 8,000 rpms. The liquid collected at the bottom, flowthrough, was then discarded. The DNA was then washed with 500 uL using the WN buffer by centrifuging for 30s at 8,000 rpms. After discarding the flowthrough, the DNA was washed again using 700 uL of WS buffer by centrifuging for 30s at 8,000 rpms. The flowthrough was discarded. Then excess wash was removed by centrifuging for 2 minutes at 14,000 rpms. To collect the DNA, it was eluted for 1 minute at 14,000 rpms using 20 uL of autoclaved water. The DNA was then placed at –20°C for storage.

BP reaction for genetic recombination

Using 3 uL of the eluted DNA, it was added to 1 uL of pDONR/ZEO and 0.5 uL of BP Clonase II. It was then left to incubate for at least 30 minutes at room temperature.

DNA Transformation

After incubation, 1 uL of the recombinant was added to another tube. TOP10 bacteria was obtained from the –80ºC freezer and placed into ice for melting. Then, approximately 50 uL of TOP10 bacteria was added to the BP recombinant. It was kept on ice for 5 minutes. Then it was heat shocked by being placed in 42ºC water for 30 seconds. Then it was placed back into ice for 5 minutes, forming the entry clones.

Grew Entry Clones

The following procedures occurred in sterile conditions. After the BP recombinant was chilled, 300 uL of LB media was added to it. The mixture incubated at 37ºC in the shaker for approximately 1 hour.

Plated Entry Clones with Zeocin

To isolate the clones, the mixture was centrifuged for 1 minute at 8,000 rpms. The clones were then resuspended using the pipette. They were added to the Zeocin plate and spread using a curved glass rod under sterile conditions. The colonies of bacteria were grown over night in a 37ºC incubator.

Growth of entry clone minicultures

The following procedures occurred under sterile conditions. Zeocin was vortexed and spun, then 2.5 uL was added to 5 mL of LB media. Pipette tips were used to collect samples from individual colonies from the Entry clone plate. The tips were then dropped into tubes containing the Zeocin and media. The tubes were then placed in a 37ºC shaker over night.

Plasmid separation

Colonies were obtained and pelleted by centrifugation for 1 minute at 14,000 rpms. The supernatant was discarded. The rest of the bacteria was extracted by repeating the previous steps until 5 mL of the media was discarded. Then Plasmid Miniprep kit was obtained. MX1 buffer kept at 4ºC was removed from the fridge, and then 200 uL was added to the Entry clone colonies. The mixture was vortexed until the colonies were resuspended. The bacterial clones were lysed, or broken, by adding 250 uL of MX2 buffer to each and inverted 5 times. The plasmids were isolated afterwards by quickly adding 350 uL of MX3 buffer to each colony. The plasmids were then inverted about 20 times. The plasmids were centrifuged for 10 minutes at 14,000 rpms. The supernatant was then transferred to separation and collection tubes. The mixture was centrifuged for 30 seconds at 8,000 rpms afterwards. The liquid flowthrough was discarded. Added to the tube was 500 uL of WN buffer I. It was then centrifuged afterwards for 30 seconds at 8,000 rpms. The flowthrough was discarded and 700 uL of WS buffer II to wash the plasmids and prevent the DNA from being eluted. The mixture was centrifuged for 30 seconds at 8,000 rpms. The flowthrough was discarded, but to rid the solution of remainder buffer, the mixture was centrifuged for an additional 2 minutes at 14,000 rpms. The flowthrough was discarded and the separating tube was then placed into an 1.5 mL microcentrifuge tube. To elute the plasmid, 50 uL of autoclaved water was added directly to the membrane of the tube. Finally, the solution was centrifuged for 1 minute at 10,000 rpms and the plasmids of the entry clones were placed in –20ºC for storage.

Creation of Destination Vector

The plasmids PMDC107, PMDC110, and PMDC111 were transformed with DB3.1 bacteria to become destination vectors. The vectors were then grown and plated on a Kanomycin and Chloramphenicol plate. The colonies were extracted, then grown in a Kanomycin solution. Once grown, the vector colonies had their DNA extracted and gel electrophoresed to confirm the size. For the electrophoresis, 5 uL of the plasmid, 1 uL of dye, and 2 uL of autoclaved. The procedures were all similar to previously described methods for promoter transformation, entry clone growth, plating, and DNA extraction.

Splice destination vector plasmids

In order to check the plasmids' sequence, they were spliced using SmaI and XhoI restriction enzymes. All the destination vector plasmids were obtained and two sets of reactions were set up. One set for the SmaI and the other for XhoI. Each received 5 uL of the plasmid, 1 uL of the respective enzyme, 1 uL of the respective buffer, and 3 uL of water. The reactions were left to incubate for 1 hour at 37ºC. Afterwards, 2 uL of dye was added to each reaction. The mixtures were then run under electrophoresis.

LR Reaction

The LR reaction combines entry clones with destination vectors using the following combination. Added first was 1.5 uL of the entry clone followed by 1.5 uL of the destination vector. Then, 1 uL of Tris buffer was added and finally 0.5 uL of LR Clonase II. The reaction was left to incubate at 37°C for an hour.

Electric Shock Transformation

TOP10 bacteria was obtained and 50 uL of the bacteria was used to transform 1 uL of the expression vector. The recombinant was then placed in an electroshock cuvette and shocked at "E. Coli1" setting until beeps signaling transfer occurs.

Expression Vector Growth

After transformation, 300 uL of LB medium was added to the vector and left to incubate in the shaker at 37°C for an hour. Once grown, the vector is then plated on kanomycin plates to grow the colonies. Both occur in sterile conditions.

Expression Vector Miniculture Growth

The following procedures are similar to those previously mentioned. The minicultures were obtained and left to grow over night.

Expression Vector Plasmid Extraction and Transformation

The following methods are similar to those mentioned earlier. First the expression vector was extracted. Then the plasmids were amplified using the GoTaq enzyme. Once a correct affirmation was received, the plasmids were transformed with Agrobacteria and plated.

Injected Arabidopsis and Nicotiana for guard cell expression

A single *Arabidopsis* specimen and 16 tobacco plants were obtained and labeled "1 through 15" and one "Full Length." Then 15 spliced sequence and 1 full length sequence bacterial recombinants were obtained. Using a p1000 Pipettman, 0.5 mL of the recombinants were each transferred to 2 mL Eppendorf tubes. Using 1 mL syringes, the bacterial recombinants were injected into the underside of 2 leaves per plant.

Methods for Determining Spermidine Concentration

Prepare 500 mL of 1/2 MS plus agar media

To prepare the media, 1.1 grams of MS salts and 0.25 grams of MES was measured and poured into a 500 mL plastic beaker. Then added 400 mL of distilled water and mixed the solution. Then the pH was adjusted by adding KOH until it was between 5.6 and 5.8. Then more water was added to reach a total volume of 500 mL. The solution was then poured into a separate bottle containing 4 grams of agar. After preparing, the solution was then autoclaved.

Sterilize seeds

Wild-type Colombia O seeds were obtained and placed into a 1.5 mL tube. The seeds were then treated with 0.05% SDS and placed on a rotator for 8 minutes. The SDS was discarded and the seeds were washed 3 times with 100% ethanol. They were then left to dry on autoclaved Whattman paper under sterile conditions.

Prepare plates

The plates were prepared using the concentrations as shown in Table 2.

Table 2. Plate Preparation Concentrations. The concentrations of Spd and ABA were added as volumes indicated in each box.

Plate Preparation Concentrations				
*Volume in uL	0 uM ABA	0.5 uM_ABA	1 uM ABA	2 uM ABA
0 mM Spd	0 0	12.5 0	25 0	50 0
0.5 mM Spd	0 125	12.5 125	25 125	50 125
1 mM Spd	0 250	12.5 250	25 250	50 250

Each box represents one plate. The top number represents voulme of ABA and the bottom represents volume of Spd both in uL.

Count seeds

A digital microscope was used to count the seeds. The seeds were classified into three groups. Stage 1 is a seed that failed to germinate. Stage 2 is a seed that began germination, characterized by the root breaking the seed coat. Stage 3 is a seed that has grown into a seedling and begins photosynthesizing.

Results for Determining Guard Cell Specific Genes

The first set of reaction set out to amplify promoters At4g24510 and At1g78530 using the GoTaq enzyme to check the functionality of the primers. Successfully amplifying the promoter region shown in fig1, then allows for amplification using the KOD enzyme necessary for cloning and DNA extraction. Receiving a positive result, as shown in fig2, the promoter At4g24510 could undergo the BP reaction, transformation, plating, and extraction. The promoter was unsuccessfully amplified so it had to undergo reduced GoTaq cycles to obtain pure strands of DNA, purify it, then amplify again using the KOD enzyme.
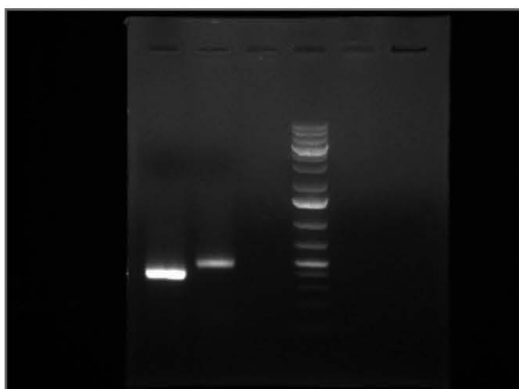
Figure 1.

2010-06-03 – gotaq – 144 145 – 156 157. The promoters At4g24510 and At1g78530 and At1g78530 were successfully amplified using the GoTaq enzyme.
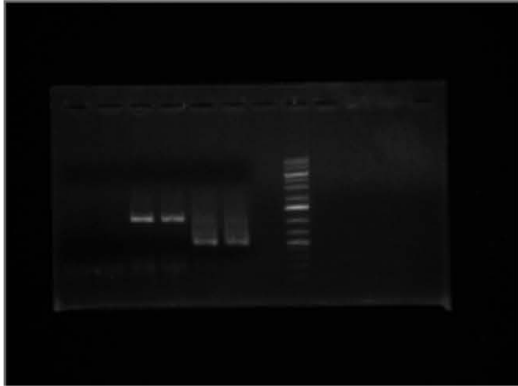
Figure 2.
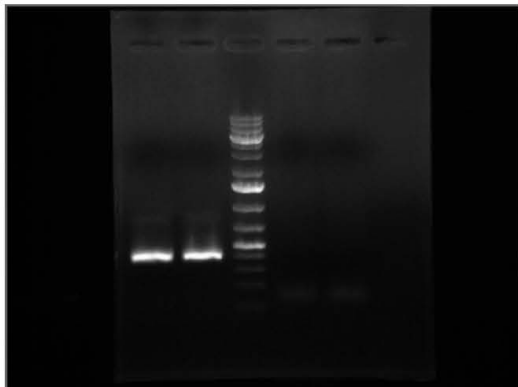
2010–06–03 – kod – 144 145 – 156 157. The promoter At4g24510 was successfully amplified using the KOD enzyme. The promoter At1g78530 was unsuccessfully amplified.

The primers for promoters At1g10060, At1g04800, At1g78530, At1g33811, At5g16240, At1g79770, and At5g19580 were checked. As shown in fig3, only 3 promoters were successfully amplified so they could then be amplified using the KOD enzyme. The others had to be redone to check for mistakes. However, fig4 shows that only promoters At1g04800 and At1g78530 were successfully amplified using the KOD enzyme, but At1g10060 was not. Thus, At1g10060 was amplified under the reduced GoTaq cycles, purified, then amplified again using the KOD enzyme seen in fig5.

Figure 3.
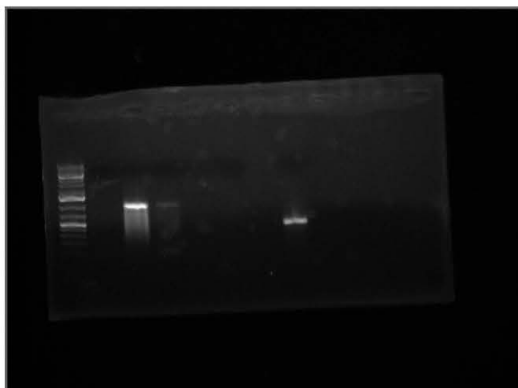
2010–06–09 – gotaq – 126 127 – 134 135 – 138 139 – 140 141 – 160 161 – 162 163 – 156 157. The promoters At1g10060, At1g04800, and At1g78530 were successfully amplified using the GoTaq enzyme, but promoters At1g33811, At5g16240, At1g79770, and At5g19580 were not.

Figure 4.

2010-06-10 – kod – 138 139 – 140 141 – 156 157. Promoters At1g04800 and At1g78530 were successfully amplified using the KOD enzyme, but At1g10060 was not.

Figure 5.
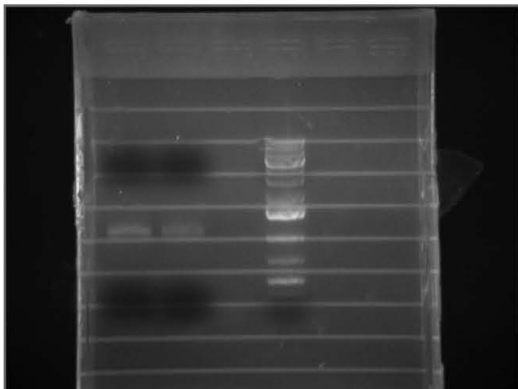
2010-06-16 – kod – 138 − 139. The promoter At1g10060 was successfully amplified by using the GoTaq enzyme for only 10 cycles, purified, then amplified using the KOD enzyme.

The next set of promoters to be amplified were At1g08810, At3g62820, At5g25840, At5g55620, At3g55500, At2g20875, and At2g21140. The primers that worked were for promoters At1g08810 and At3g62820, fig6. Those were then amplified using the KOD enzyme, but failed. Sown in fig7, the promoter At2g20875 was successfully amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme, but At5g25840, At5g55620, At3g55500 and At2g21140 were not and cloning ceased. Only promoters At1g08810 and At3g62820 were successfully amplified using the KOD enzyme, fig8, so they could undergo the BP reaction, transformation, plating, and extraction. The promoters At1g08810 and At3g62820 were amplified successfully under reduced GoTaq cycles having failed KOD amplification, fig 9, so cloning continued. However, promoters At5g55620 and At2g20875 were still unsuccessfully amplified and cloning ceased.
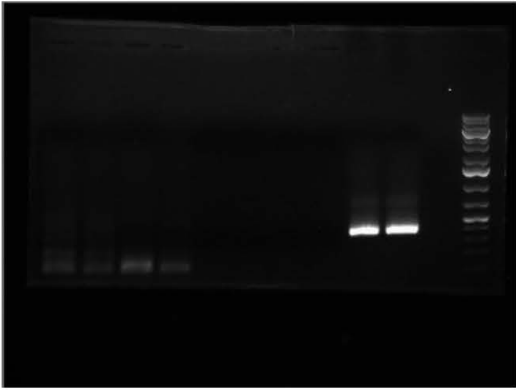
Figure 6.
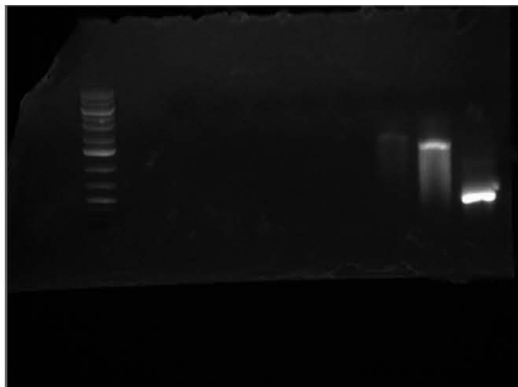
2010–06–23 – gotaq – 124 125 – 128 129 – 130 131 – 132 133 – 136 137 – 142 143 – 146 147. The promoters At1g08810 and At3g62820 were successfully amplified using the GoTaq enzyme. The promoters At5g25840, At5g55620, At3g55500, At2g20875, and At2g21140 were not.

Figure 7.
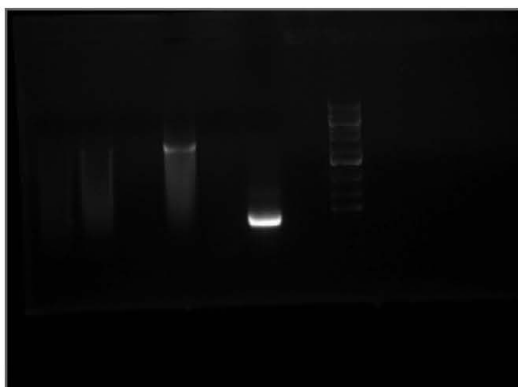
2010–06–28 - gotaq - 130 131 - 132 133 - 136 137 - 142 143 - 146 147 - ctrl 144 145. The promoter At2g20875 successfully amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme, but At5g25840, At5g55620, At3g55500 and At2g21140 were not.
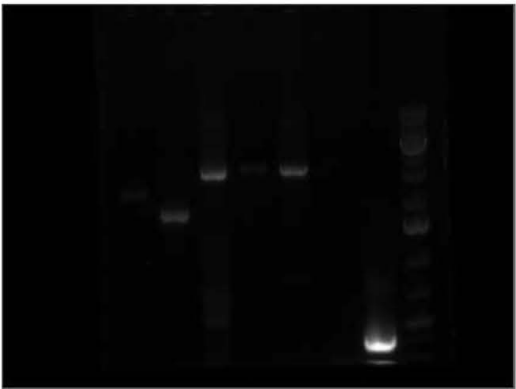
Figure 8.

2010–06–28 - kod - 124 125 - 128 129 - ctrl 144 145. The promoters At1g08810 and At3g62820 were successfully amplified using the KOD enzyme.
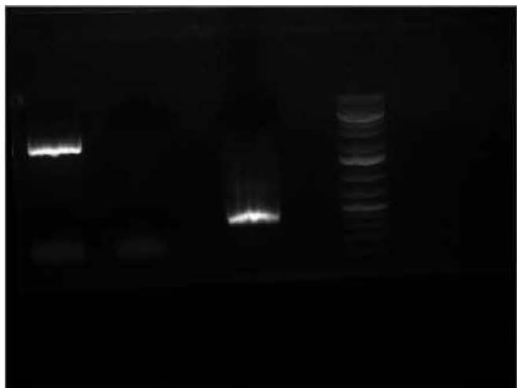
Figure 9.

2010-06-30 – kod – 124 125 – 128 129 – 132 133 – 142 143 – ctrl 144 145. The promoters At1g08810 and At3g62820 were successfully amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme, but At5g55620 and At2g20875 were not.

The primers for promoters At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, and At1g62400 were next to be checked. Shown in fig10, promoters At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, were successfully amplified using the GoTaq enzyme, but At1g62400 was not. Since none of the promoters that worked using the KOD enzyme, fig 12, they all were run using reduced GoTaq cycles. Shown in fig11, fig12, and fig13, the promoters At5g55620 and At2g21590 were successfully amplified using reduced GoTaq enzyme cycles and cloning for promoters At2g20875, At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, and At1g62400 ceased.
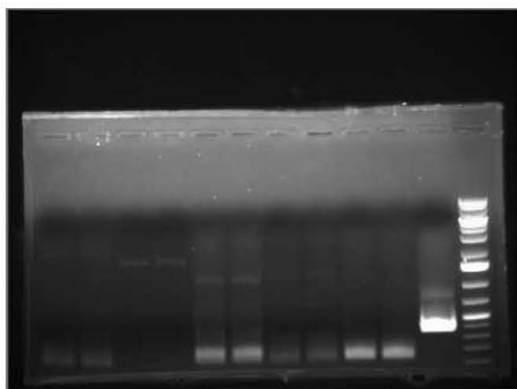
Figure 10.

2010-07-13 – gotaq – 148 149 – 150 151 – 152 153 – 154 155 – 164 165 − 158 159 – ctrl 144 145. The promoters At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, were successfully amplified using the GoTaq enzyme, but At1g62400 was not.
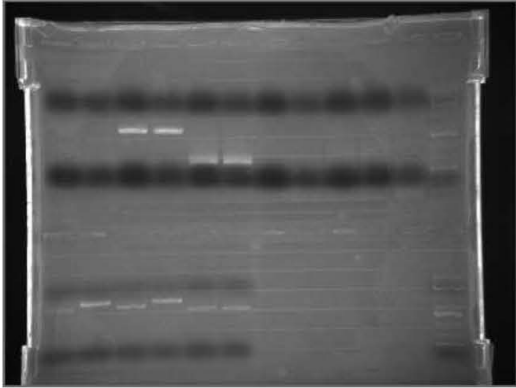
Figure 11.

2010–07–13 – kod – 132 133 – 142 143 – ctrl 144 145. The promoter At5g55620 was successfully amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme, but At2g20875 was not.

Figure 12.

2010–07–13 – kod – 148 149 – 150 151 – 152 153 – 154 155 – 164 165 – ctrl 144 145. The promoters At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, and At1g62400 were unsuccessfully amplified using the KOD enzyme.
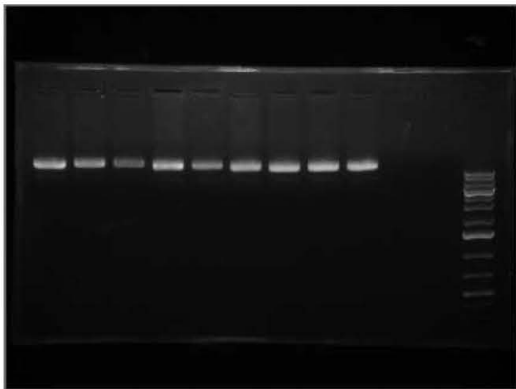
Figure 13.

2010–07–21 – kod – 148 149 – 150 151 – 152 153 – 154 155 – 164 165 – ctrl 144 145. Promoters At2g04570, At2g21590, At2g46720, At3g23840, At1g75880, and At1g62400 were inefficiently amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme.
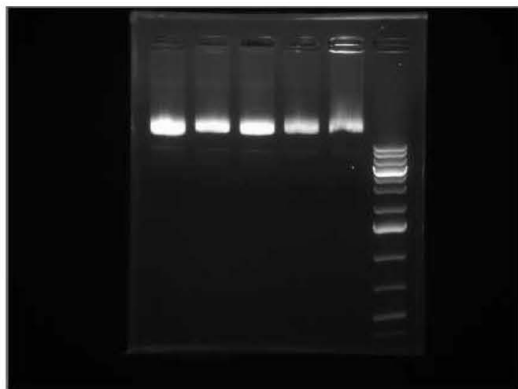
Figure 14.

2010-07-23 – kod – 148 149 – 150 151 – 152 153 – 154 155 – 164 165 – ctrl 144 145 and plasmids. Shown at the top, the promoter At2g21590 was were successfully amplified using the GoTaq enzyme for 10 cycles, purified, then run using KOD enzyme, but At2g04570, At2g46720, At3g23840, At1g75880, and At1g62400 were not. Shown on the bottom, the entry clone plasmids pDPE001, pDPE002, pDPE003, pDPE004, pDPE005, and pDPE006 were successfully extracted.

The promoters At4g24510, At1g04800, At1g78530, At1g10060, At1g08810, At3g62820 were successfully converted into entry clones corresponding to the names pDPE001-6 shown in fig14. These were then sent to have their DNA checked for any mistakes. Only pDPE001-4 were correct. These could then be made into expression vectors, but first the Destination vectors had to be made. Destination vectors named pDPD001-3 were made combining DB3.1 and PMDC107, PMDC110, and PMDC111 shown in fig15 and fig16. They were then checked for correctness by splicing. Shown in fig17 and fig 18 the destination vectors worked, but only with the XhoI restriction enzyme to splice them. It was only necessary to chose one destination vector to make the expression vectors, so pDPD001 was chosen. So pDPD001 was used for pDPE001-4 to make the expression vectors pDPX001-4 successfully, fig19. All of the results are summarized and explained in Table1.
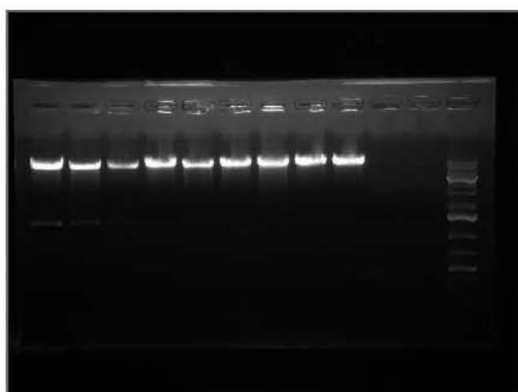
Figure 15.

2010-07-29 – pDPD001-3, A–C. The destination vector plasmids pDPD001-3, A–C were successfully extracted, but were not concentrated enough.

Figure 16.

2010-07-30 – pDPD001-3, A–C. The destination vector plasmids pDPD001-3, A–C were concentrated producing bright bands.

Figure 17.
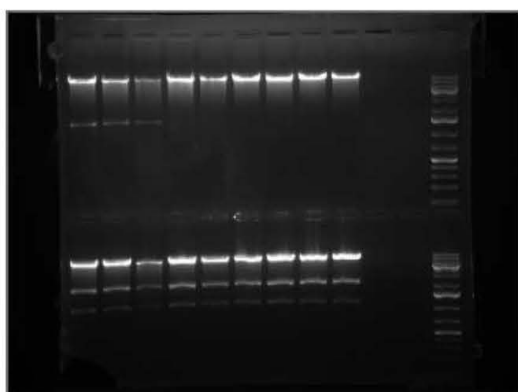
2010-08-04 – pDPD001-3, A–C XhoI and SmaI. Shown at the top, the restriction enzyme SmaI failed to splice the destination vectors pDPD001-3, A–C. Shown at the bottom, the restriction enzyme XhoI successfully spliced the destination vectors pDPD001-3, A–C.

Figure 18.

2010-08-06 – pDPD001-3, A–C SmaI. SmaI insufficiently spliced the destination vectors pDPD001-3, A–C.

Figure 19.
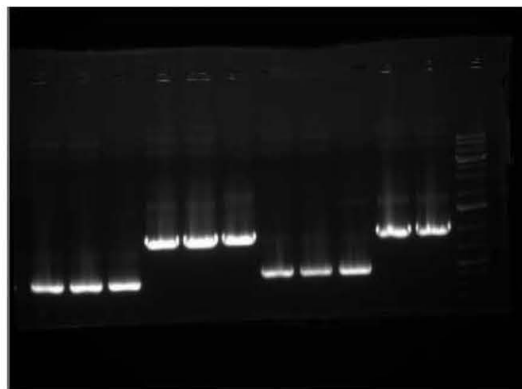
2010-08-19 – pDPX001-4, A–B. The plasmids for expression vectors pDPX001-4, A–B were successfully extracted.

Table 1.

Preliminary Results for Guard Cell Preferential Genes				
Protein	AGI number	Primer F	Primer R	Results
GDSL–motif lipase family protein	At1g33811	126	127	Primers Don't Work
Expressed protein	At5g25840	130	131	Primers Don't Work
Acyl ACP (acyl carrier protein) desaturase	At5g16240	134	135	Primers Don't Work
Expansin (EXP6)	At3g55500	136	137	Primers Don't Work
Hydroxyproline rich protein	At2g21140	146	147	Primers Don't Work
Expressed protein	At1g79770	160	161	Primers Don't Work
Putative protein	At5g19580	162	163	Primers Don't Work
Expressed protein	At5g55620	132	133	Being Sequenced
EPF1	At2g20875	142	143	Being Sequenced
MYB60	At1g08810	124	125	Wrong Sequence
Pectin esterase homolog	At3g62820	128	129	Wrong Sequence
Branched chain amino acid transamidase (BCAT1)	At1g10060	138	139	Agrobacterium Transformation
Glycine rich protein	At1g04800	140	141	Agrobacterium Transformation
CER2	At4g24510	144	145	Agrobacterium Transformation
Receptor protein kinase, putative	At1g78530	156	157	Agrobacterium Transformation

Preliminary Results for Guard Cell Preferential Genes. The promoters that did not continue with cloning are in the "primers don't work" column. The promoters awaiting DNA sequence confirmation are categorized as "being sequenced." The promoters that had incorrect DNA sequences were categorized as "wrong sequence." The promoters that were converted into expression vectors and awaiting to be transferred into plants for analysis are categorized as "agrobacterium transformation."

Preliminary Results for Determining Spermidine Concentration

After counting the three stages of seeds, fig20, fig21, and fig22, the percentages of surviving seeds were counted for the various Spd and ABA concentrations. Fig23 shows that as the percentage of surviving seeds for all Spd concentrations decreased as the concentration of ABA increased. However, when seeds were treated using 0.5 mM Spd, over 85 percent of the seeds survived initially. For those treated with 0 mM Spd, 70 percent of the seeds survived initially. For those treated with 0 mM Spd, only 60 percent of the seeds survived initially.
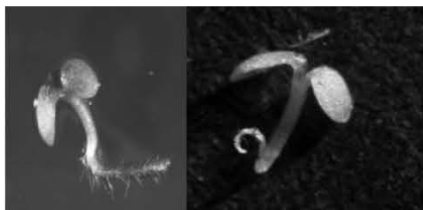
Fig20.
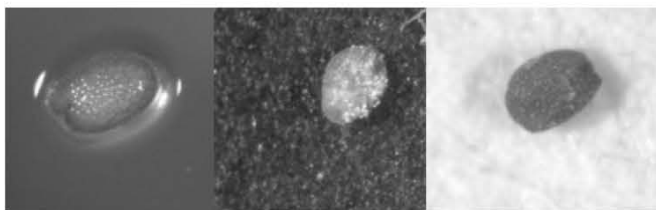
Stage 1 seeds. These seeds failed to germinate.
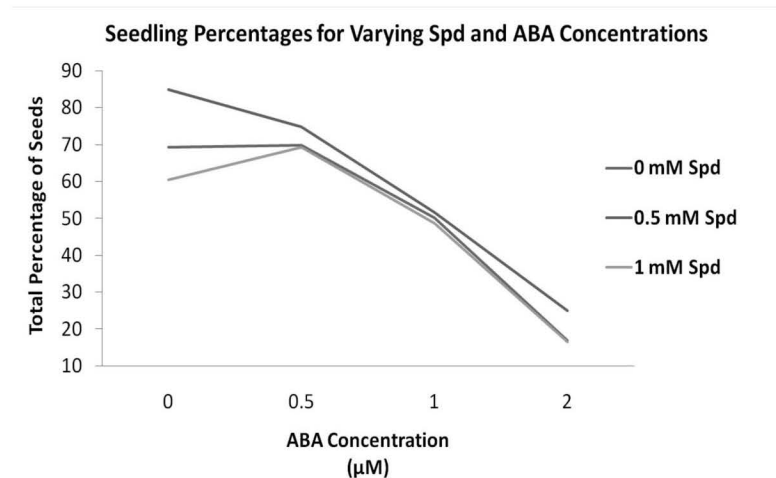
Fig21.

Stage 2 seeds. These seeds germinated, which is shown via the root breaking the seed coat.

Fig22.

Stage 3 seeds. These seeds have matured into seedlings that undergo photosynthesis.

Figure 23.

Seedling Percentages for Varying Spd and ABA concentrations.

For seeds that were treated under 0 mM concentration of Spd, as ABA concentration increased, the seeds's survival dropped from 70 to 17 percent. For seeds that were treated under 0 mM concentration of Spd, as ABA concentration increased, the seeds's survival dropped from 85 to 25 percent. For seeds that were treated under 1 mM concentration of Spd, as ABA concentration increased, the seeds's survival dropped from 60 to 16 percent.

Discussion

My research is still ongoing, so I will discuss future research aspirations. Having transformed the expression vectors into Agrobacteria, the next aspect of my research is to determine the guard-cell specificity of the promoter sequences. Thus, I must perform GUS reporter assays and identify regulatory regions in the promoters to determine gene expression patterns in plants.

Although there has been little data collected regarding Spermidine concentrations, some conclusions can still be drawn. The data does support my hypothesis that Spd will increase seed viability. By successfully inhibiting the phase transitions from embryonic to germinative growth, Spd was able to keep the seeds alive. Data showed that in the presence of Spd the percentage of surviving seeds greatly increased. Spd concentration of 0.5 mM worked the best under changing conditions, setting a standard for future studies using Spd concentration. However, too much Spd added to seeds was toxic to seeds. Data also showed that in excess ABA and Spd concentrations, seeds do not germinate. This finding supports previous research that the ABA pathway is far too overpowering and complex for drugs to fully prevent. However, the use of Spd will be able to significantly cut the number of dying seeds. Thus, Spd had to be kept under 1 mM concentration. In the future it is necessary to first replicate these findings. Afterwards, the concentrations of Spd can be more actively studied for determining the best concentration of Spd to use.

Conclusion

By finding the genes responsible for stomatal guard cell closure involved in the ABA pathway it will be possible to control when stomata open and close. Plants will be able to better regulate gas exchange processes that contribute to the 95 percent of water loss in plants. Having the ability to stop the single most destructive process detrimental to plant survival will allow even common plants to withstand the driest of droughts. Being able to develop a faster method for identifying regions in the promoter that encodes functionality is crucial for accelerating the progress of this important study.

Chemically, inhibiting phase transitions from embryonic to germinative growth using Spd gives the new ability to manipulate seed germination. By having a standard for the treatment of seeds using Spd, complete seed germination control is possible in the near future. Thus, seeds can be treated to endure the coldest and harshest of winters long enough for favorable growing conditions in the spring.

Combining both the genetic and chemical aspects of the ABA pathway which is responsible for plants' response to environmental stress is necessary to even begin to comprehend this process. By further understanding these two seemingly unrelated responses involving stomatal closure and seed germination provides further insight into the still largely unknown Abscisic Acid signaling pathway to make plants impervious to environmental disasters. Thus, plants necessary for all peoples across the world to survive and thrive on will be accessible to all creating much a greener place for us to live.

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