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Removal of arabinogalactan protein 31 from *Arabidopsis* leads to a decrease in expression of ABA signaling target gene RD29B, while ABA signaling has no significant effect on OLE1 transcription

Abstract:

Due to the warming climate, it is crucial scientists understand stress tolerance mechanisms to help prevent decrease in yield of agricultural crops. Abscisic acid (ABA) is a plant signaling hormone involved in regulating a stress response due to drought, cold weather and infection in many plants and is secreted to start a signal cascade to ultimately activate target genes. Our lab focused on studying the effects of the ABA response after removing Arabinogalactan protein 31 (AGP31) in our model system Arabidopsis, a gene recently known to decrease the influence of the ABA response if not present in the genome. We wanted to understand how ABA and AGP31 work together by testing how the AGP31 mutants differed from the wildtype ABA response of target gene regulation. Three lines of Arabidopsis plant tissue was cultured: Ler0 (wildtype for AGP31), 6134 (mutant for AGP31), and R2-4 (rescued line, AGP+/+). After treating each line with an ABA solution containing ethanol or control lines of EtOH only, RNA was isolated and exhibited accepted purity and intactness via spectroscopy and gel analysis. cDNA was created from our RNA samples using reverse transcriptase. We designed primers for the OLE1 gene, a gene involved in seed growth, to quantify mean fold change in gene expression via qPCR. We performed qPCR with RD29B gene as a known ABA target gene and OLE1 gene, a gene that is upregulated during seed development but unknown to whether ABA treatment influences transcription regulation . The qPCR results revealed little increase in OLE1 gene expression between EtOH and ABA treated lines in both Ler and 6134 genotypes, while both Ler and 6134 genotypes exhibited an increase in mean fold gene expression in ABA treated lines for RD29B. This suggests that ABA and the AGP31 gene interact subtly to produce robust transcription of ABA target gene, but ABA still targets genes despite lack of AGP31. OLE1 transcription was observed to not be significantly influenced by the ABA pathway. Further testing of how AGP31 increases gene expression of RD29B and how OLE1 gene expression is regulated is needed to understand stress tolerance mechanisms in Arabidopsis for agricultural use.

Results

Experimental Set-Up

To understand how AGP31 protein is involved in the ABA response in Arabidopsis, we designed our experiment to compare three different lines of Arabidopsis. The wildtype line, referred to as “Ler0”, the AGP31 mutant line, referred to as “6134”, and the rescued AGP31 line, referred to as “R2-4”. The 6134 mutants were made by adding transposon AcDs element into a wildtype sample, allowing for the element to move around the genome. The AC element was then crossed out of the plant line, and samples were sequenced to find samples with Ds disruption of exon 2 in our target AGP31 gene. Our rescued lines were created by taking mutant lines and adding a wildtype AGP31 gene back into the sample.

Each biological replicate (BR) was grown on its own plate of growth media and given supplemental sunlight for 13 days. We grew six biological replicates per genotype (three BRs for each plate media, ABA treatment or ethanol control, as explained below). We grew several BR of the same genotype to account for natural variation seen within a normal population, as well to account for handling effects of the samples. On the 13th day, each sample was transferred to either a 10 uM ABA containing plate or an ethanol control plate via forceps, and placed under the same supplemented sunlight for 3 hours. We then harvested .1g samples from each replicate for our experimental analysis.

Table 1: Labeling of Biological Replicates system. Each BR also contains a number to uniquely identify it

Genotypes	Treatment	Abbreviation
Ler	Ethanol control	LE
Ler	ABA treatment	LA
6134	Ethanol control	6E
6134	ABA treatment	6A
R2-4	Ethanol treatment	RE
R2-4	ABA treatment	RA

RNA Purification from Biological Replicates

Isolation and purification of RNA from each BR was needed to ultimately quantify gene expression in each experimental line. The cell tissue samples from the process described in the previous section were individually frozen, lysed open using TissueLyser, and resuspended in buffer solution. All samples were then incubated to room temperature, and RNA isolation by Qiagen kit was performed. After isolation, only mRNAs and rRNAs (RNA>200bp) are present in the solution. After the total RNA prep for each sample was complete, purity of each sample was checked via spectroscopy and gel electrophoresis analysis. Spectroscopy analysis was measured using a Nanodrop spectrophotometer to check for quality and concentration of RNA from each BR. Each total RNA prep was analyzed by pipetting 1-1.5ul of the sample onto the nanodrop, which then measured the absorbances of the sample by emitting light. Our spectroscopy results revealed a concentration of 464.8 ng/ul and 474.3 ng/ul yield for my samples 6E11 and 6A1, respectively. Readings for residual protein contaminants were measured at 260/280 revealed 2.12 and 2.03 for 6A1 and 6E11 samples, respectively, all above necessary 1.8 value. Readings for residual salt and carbohydrates contaminants were measured at 260/230 revealed 2.52 and 1.97 for 6A1 and 6E11 samples, respectively, both between the necessary 1.8-2.0 value.Table 2 summarizes all the results from the lab. Repurification for these samples was not necessary.

Table 2: Summary of RNA Purity Results from Nanodrop Spectrophotometer

BR sample name	Concentration (ng/ul)	260/280 (residual protein)	260/230 (residual salts, carbs, etc)
6A1	474.3	2.12	2.52
6E11	464.8	2.03	1.97
LA1	406.1	2.17	2.24
LA2	540	2.18	2.33
LA4	422.1	2.14	2.49
LA6	346.9	2.16	2.4
LA10	502.9	2.21	2.66
LE1	300.4	2.18	2.22
LE5	645.1	2.21	2.46
LE7	593	2.18	2.48
LE8	394.4	2.14	2.48
LE10	874.9	2.19	2.51
6A2	525	2.19	2.34
6A3	643	2.2	2.44
6A4	311.4	2.21	2.94
6A5	431.4	2.14	2.75
6E2	414.7	2.15	2.79
6E14	666.1	2.21	2.54
6E16	446.1	2.13	1.96
6E13	501.4	2.22	2.57
RA1	515	2.18	2.57
RA6	386	2.17	2.41
RA8	417.8	2.12	2.75
RA9	526.1	2.26	2.6

A gel analysis was performed to check for RNA intactness (lack of degradation) . An aliquot of each total RNA prep was mixed with ethidium and loading dye and heated to denature double stranded RNA to single stranded. Each sample was then run on a denaturing agarose gel containing formaldehyde to maintain single stranded nature of RNA during the gel electrophoresis. The gels were then analyzed

under a UV light box and checked for bright bands at the 28s and 18s mark. Both 6A1 and 6E11 exhibit bright, crisp bands at these locations, with a little smearing underneath. The 28s band looks twice as bright as the 18s band, as should be expected due to 1:1 stoichiometry in the cell, but almost twice the size in molecular weight. The smearing indicates low molecular weight RNAs that could be present in the sample due to RNA degradation. Figure 1 depicts the gel results.

Figure 1: Gel Analysis Results

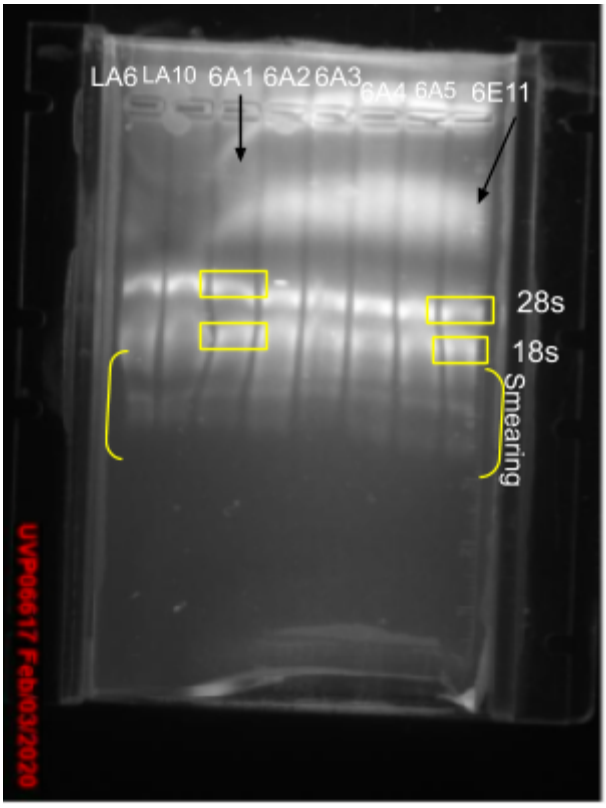


Figure 1 depicts gel results. The two yellow boxes in each line indicate the 28s and the 18s rRNAs expected to be present and clear. Sample 6A1 and 6E11 are labeled.

cDNA Synthesis

Each total RNA prep for each BR was then used to make cDNA. The sample cDNA collected from each BR is required for qPCR, which quantifies the gene expression in each experimental condition. For cDNA synthesis, each RNA prep was prepped first by adding DNAse to remove most residual DNA, then EDTA to inactivate the DNAses then finally by removing 1ug from each experimental group. These aliquots of each sample did not receive any reverse transcriptase (RT) enzyme, and served as a control group for each experimental condition to determine if any residual DNA might have served as an unwanted primer. Experimental samples of 1ug from each BR RNA prep were then created by adding in RT, then all samples received dNTPs, random 6 bp primers, and a compatible buffer. All samples were placed in the Biorad PCR Cycler , in which RNA was converted to DNA copies.

Designing Primers for qPCR Analysis

For the qPCR experiment, our lab was interested in quantifying relative gene expression of OLE1 gene for each experimental condition. Four sets of forward and reverse primers were chosen for OLE1 gene using primer designer software PerlPrimer. Melting curves, amplification curves and standard curves were generated for each set of primers after PCR using cDNA samples. Both the melting curves and the amplification curves depicted predicted normal results. Primer set 4 was chosen for the following qPCR experiment because it yielded the best replication percentage (slope of -3.14) by plotting threshold cycles against log[cDNA]. No template controls (NTC) (samples that did not contain cDNA) yielded the expected results of a flat CT curve. The standardization curve for all four primers are depicted in Figure 2.

Figure 2: Standardization graphs for OLE1 primers

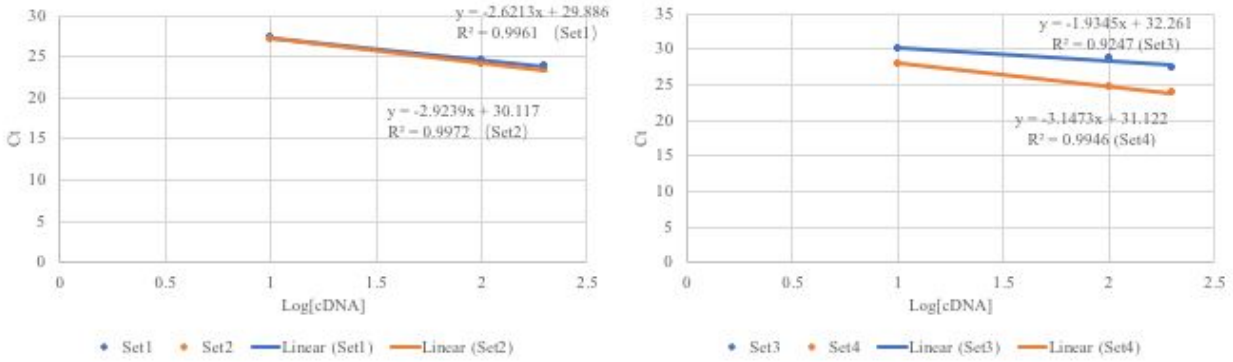


Figure 2 represents all four primer sets created and tested for the OLE1 gene. In the first graph on the left, the blue represents set1 primers, and orange represents the set2 primers. In the graph on the right, blue represents the set 3 primers and orange represents set 4 primers (the primer set we chose for qPCR)

qPCR experiment:

After creating cDNA and selecting OLE1 primers, qPCR was carried out for all of our BRs. Each BR amplified three different genes using three different primers. The first gene, actin, was used as a control for gene expression in this experiment. Actin is a gene known to be expressed at constant levels in Arabidopsis during ABA and non-ABA exposure. These results yielded us a constant to generate our delta CT values for our other two genes of interest. We also measured gene expression of RD29B, a gene previously known to have higher expression levels during wildtype ABA exposure. These results were used to make sure our wildtype Ler0 samples were behaving as normal, with higher gene expression after ABA exposure, as well as to understand the interplay between AGP31 during ABA exposure on a known ABA target gene. Our third gene, OLE1, was measured to understand how ABA, AGP31 and OLE1 gene regulation all work together.

Mean Fold Change in Gene Expression

After obtaining raw CT values for each BR for each gene via qPCR sybr green method, averages for each treatment and genotypes we calculated. A set of calibrator samples were labeled for comparisons of mean fold gene expression, shown in Table 3. After calculating the difference between delta CT values of calibrator versus test sample, individual gene expression values (2^delta delta CT) were averaged together to yield mean fold change in gene expression for each of the comparisons. Quantitative results

from each comparison are shown in Table 4, as well as graphs A-H below. Interpretation of results are located in the discussion section of the report.

Results of qPCR

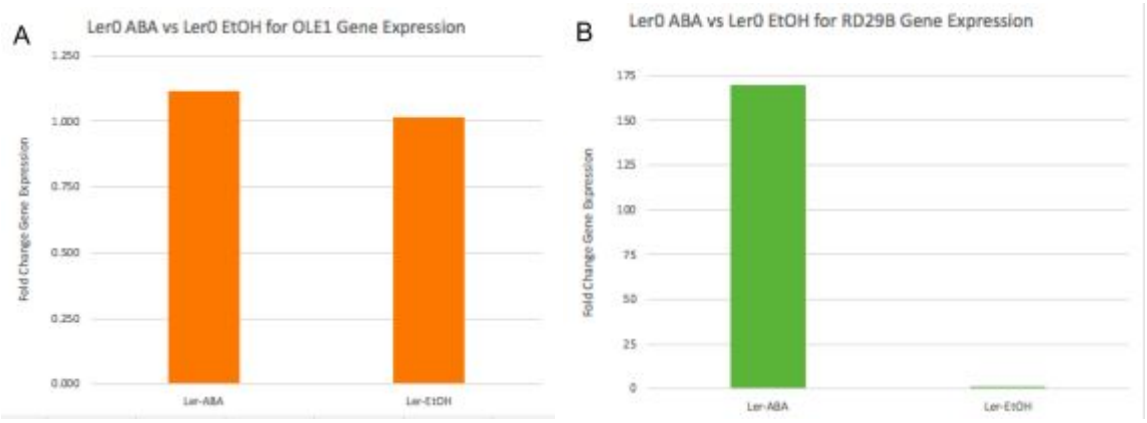
Table 3: calibrator and sample BRs for each graph comparison

Graph Name	Gene of Interest	Test Sample	Calibrator
A	OLE1	Ler-ABA	Ler-EtOH
B	RD29B	Ler-ABA	Ler-EtOH
C	OLE1	6134-ABA	6134-EtOH
D	RD29B	6134-ABA	6134-EtOH
E	OLE1	6134-EtOH	Ler-EtOH
F	RD29B	6134-EtOH	Ler-EtOH
G	OLE1	R2-4 ABA	R2-4 EtOH
H	RD29B	R2-4 ABA	R2-4 EtOH

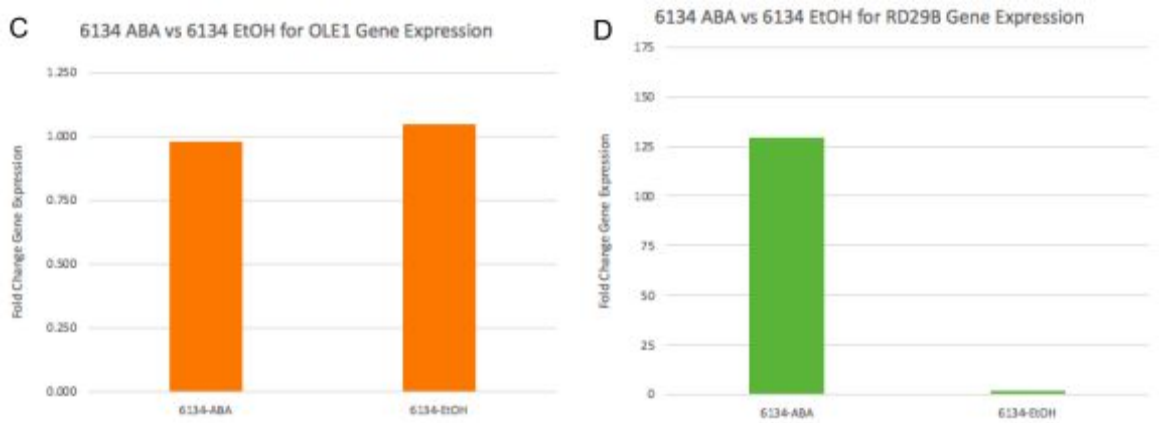
Table 4 : Relative gene expression comparisons

Relative Gene Expression				
Graph Name	Gene of Interest	BR groups	Gene expression	SEM (of 2^-ΔΔCT)
Graph A	OLE1	Ler-ABA	1.114	-
		Ler-EtOH	1.014	-
Graph B	RD29B	Ler-ABA	169.4908322	-
		Ler-EtOH	1.104805008	0.361904956
Graph C	OLE1	6134-ABA	0.977	0.727
		6134-EtOH	1.044	-
Graph D	RD29B	6134-ABA	129.789739	73.28567928
		6134-EtOH	2.09451617	-
Graph E	OLE1	6134 EtOH	9.875988908	-
		Ler0 EtOH	1.014064601	-
Graph F	RD29B	6134 EtOH	6.213051774	-
		Ler0 EtOH	1.104805008	0.361904956

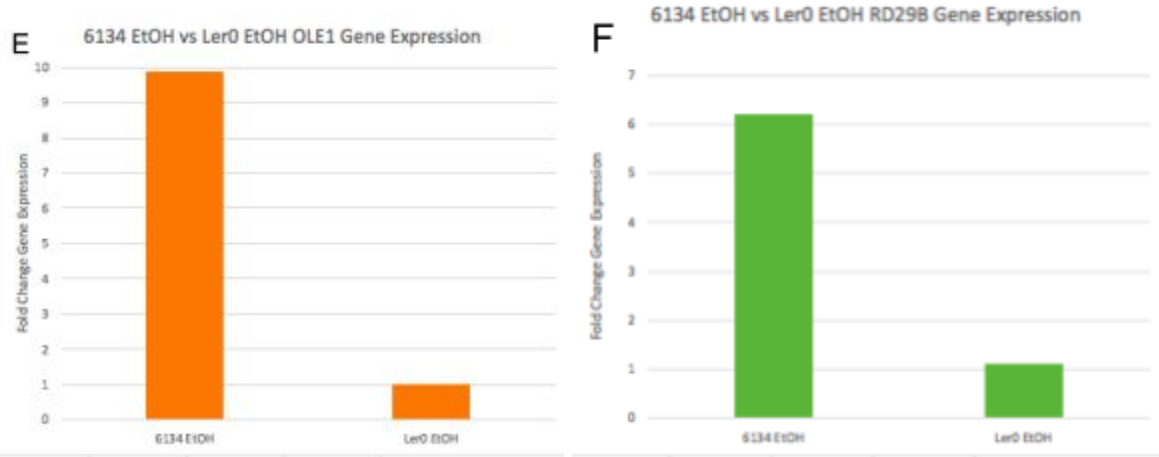
Only treatment types with 3 valid BR values were used to calculate SEM



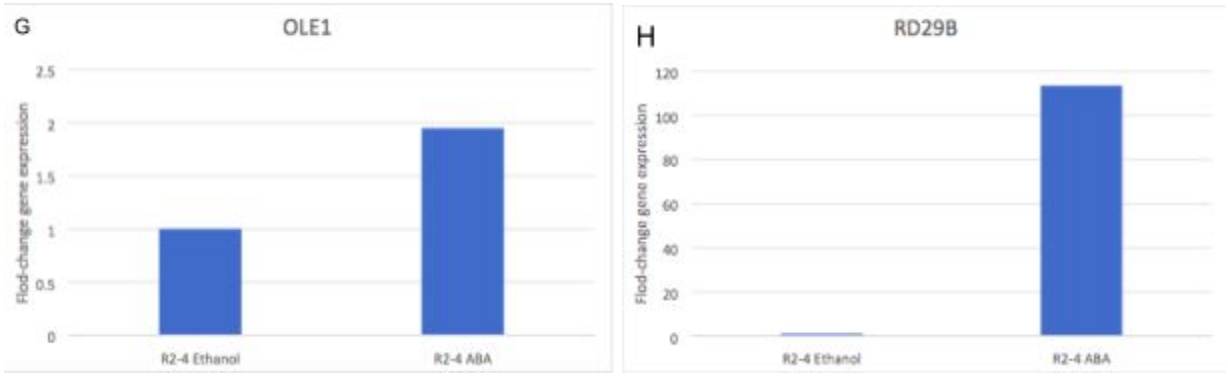
Graphs A and B depict the mean fold gene expression changes between Ler0 ABA (test sample) and Ler0 EtOH (calibrator) for OLE1 (left) and RD29B (right) genes. Graph A shows a fold expression fold change of .114 from EtOH to ABA Ler0 treatment of OLE1. Graph B shows a 168.3 fold change in gene expression from EtOH to ABA Ler0 treatment of RD29B.



Graphs C and D depict the mean fold gene expression changes between 6134 ABA (test sample) and 6134 EtOH (calibrator) for OLE1 (left) and RD29B (right) genes. Graph C shows a fold expression fold change of -.067 from EtOH to ABA 6134 treatment of OLE1. Graph B shows a 127.69 fold change in gene expression from EtOH to ABA 6134 treatment of RD29B.



Graphs E and F depict the mean fold gene expression changes between 6134 EtOH (test sample) and Ler0 EtOH (calibrator) for OLE1 (left) and RD29B (right) genes. Graph E shows a fold expression fold change of 8.86 from EtOH Ler0 to 6134 EtOH of OLE1. Graph F shows a 5.11 fold change in gene expression from EtOH Ler0 to 6134 EtOH of RD29B.



Graphs G and H depict the mean fold gene expression changes between R2-4 ABA (test sample) and R2-4 EtOH (calibrator) for OLE1 (left) and RD29B (right) genes. Graph G shows a fold expression

change of roughly 1 from EtOH to ABA R2-4 treatment of OLE1. Graph H shows roughly a 109 fold change in gene expression from EtOH to ABA R2-4 treatment of RD29B.

Discussion:

The ABA pathway is an essential response for Arabidopsis and other plants to preserve resources in the face of adversity (i.e drought, pathogens, cold temps). Our lab decided to focus on the possible interaction arabinogalactan protein 31 (AGP31) in the ABA pathway response because it has been previously noted that AGP31 is upregulated after ABA treatment. AGP31 is normally expressed in the vascular system of the plant, the same locations in which ABA molecules travel to other cells to signal a stress response.

The RNA purification steps produced the desired results. All samples were checked for quality and quantity of the correct RNAs via gel electrophoresis and spectroscopy. All samples were within the desired range (1.8-2.1+) for absorbance ratio 260/280, which indicates if any samples had too much residual protein. Samples were also within the desired range (>2.3) for the absorbance ratio 260/230, which if too low indicates too much residual carbohydrates, salts or other contaminants left over from the RNA purification steps. Gel results from gel electrophoresis check also produced the desired results for each sample. Gels from each sample indicated two bright bars at 28s and 18s, which was to be expected. Some samples contained more smearing at under the 18s bar, but that may be attributed to other small organelles or RNAs that left a small signal in the gel. Overall, RNA purification produced clean and viable samples for qPCR.

Each sample was converted from RNA to cDNA using reverse transcriptase. Negative controls for each treatment type (-RT) were measured and compared to +RT samples. This allowed us to measure how much residual DNA was leftover in our samples after DNAse treatment to compare qPCR output in the next section. Primers were designed for the OLE1 gene for our qPCR experiment. Amplification and melting curves were examined for primers to make sure primers did not self anneal as well as proving steady amplification each round of PCR. CT values for different concentrations of primer were recorded and graphed for each set of possible primers for OLE1. Primer set 4 was chosen because it proved to be the best at amplifying across all concentrations with a slope of -3.1, closest to the perfect amplification slope of -3.3.

Our lab focused on quantifying the expression of three primers, as explained in the results section. Actin, a stably expressed gene under many conditions,, including ABA exposure, was chosen as a control for computing our delta CT values. This accounts for the pipetting variation, as well as sample quality variation between our samples. RD29B was chosen because it is an accurate measure of ABA stress response. RD29B is a known target gene of ABA stress pathway, and therefore a good measure to examine ABA effects with and without AGP31 gene. Oleosin 1, (OLE1) is a gene involved in seed oil content. In normal wildtype Arabidopsis, OLE1 is expressed in high levels during seed development, and decrease in expression levels in 10-13 day old seedlings. Since the ABA pathway is heavily involved in seed development during times of stress, it seemed necessary to look at how the expression of an imperative seed development gene was affected by ABA exposure.

Our PCR quantified CT values based on the sybr green method. Like normal PCR, this method requires template DNA (cDNA in our case), primers, dNTPs, DNA pol. In addition to these materials, sybr green dye is added to the mixture. Syber green binds to the dsDNA generated and strongly fluoresces above unbound levels, allowing an accurate measure of fluoresces to equate to dsDNA copies of our target genes. Unfortunately, some of our test samples produced undetermined CT values, thus limiting our mean fold gene expression changes or each treatment to an even smaller sample size. Our -RT controls also produced an undetermined CT value, but this was to be expected. If no cDNA was present in the sample in the first place, we can attribute all leftover genomic DNA in our samples after cDNA creation to have a negligible effect on our qPCR results. Our No Template Controls also run in the qPCR experiment also produced an expected undetermined CT result. This helped us determine that little to none primer dimers were formed, also not a source of confounding error in our test sample qPCR results. Mean fold gene expression results and comparisons are explained below.

Examining graph A (Ler ABA/EtOH for Ole1) only a small +.1 increase in mean fold gene expression of OLE1 expression in ABA treated wildtype lines compared to EtOH only wildtype lines is depicted. Graph B serves as a control to make sure the experiment was carried out correctly because we received anticipated results of a large increase (+168.386) in mean fold gene expression of known ABA upregulated gene RD29B. Comparing graph A to B, it is evident that ABA treatment in wildtype Ler lines has little effect on the upregulation of OLE1 gene expression. Looking at the AGP31 mutant lines in graph D, which examines RD29B gene expression in 6134 lines for both ABA treated and EtOH treated BRs, it is evident that while there still is an +127.69 increase in mean fold gene expression in ABA treated vs EtOH treated, the increase in expression is about 24% less (about 40.691 mean fold gene expression difference) than the increase in gene expression shown in Graph B. This indicates that while ABA is still targeting its known RD29B target gene and increasing its expression in the 6134-ABA line, it is being hindered compared to wildtype expression of RD29B in Ler-ABA line.This difference in gene expression is most likely due to the loss of AGP31 in the 6134 BRs, but it is also possible this conclusion is random chance. Repetition of this experiment with more BR samples revealing the same results would increase the confidence that loss of AGP31 is the main cause in the decrease in RD29B gene expression.

Graph C depicts the mean fold change in gene expression of OLE1 gene for 6134 ABA and EtOH lines. There is a minimal .067 decrease in gene expression in OLE1 from EtOH to ABA BRs, suggesting that the decrease is due to chance rather than treatment type. The results of Graph C resemble the results in graph A, further suggesting that ABA signalling gene is not involved in the regulation of OLE1 gene expression. Once again, further repetitions of this experiment with more BR samples would increase the confidence that ABA treatment is not involved in OLE1 regulation.

Graph E depicts the mean fold change in gene expression between EtOH only lines for both Ler and 6134 BRs. Comparing these two treatments allows us to examine the increase in OLE1 expression that is not due to ABA signaling, and rather the influence of AGP31 on gene expression. There is a 5.19 mean gene fold expression increase in OLE1 expression in the 6134 treatment group compared to the Ler group. While there is an increase in OLE1 gene expression, the increase is minute compared to the increase in gene expression in RD29B seen in both graphs B and D. Increasing the sample size of the experiment would help determine if this increase is significant.

Graph F shows the mean fold change in gene expression of gene RD29B for EtOH only treatment for Ler and 6134 lines. This graph resembles graph E, showing a 5.108 increase in mean fold gene expression in 6134 compared to Ler. This graph somewhat contradicts our findings seen in graphs B and D. Graph B shows an increase in RD29B for Ler ABA treatment that is +39.7 mean fold change higher than the increase seen in 6134 ABA. As previously stated, it is known that ABA treatment increases RD29B expression, and this suggests both that AGP31 may be hindering the RD29B robust expression. However, having both genotypes treated with only EtOH, graph F suggests that the increase in RD29B gene expression is not solely due to ABA treatment, but also lack of AGP31. This may suggest that while ABA + AGP31 certainly increases RD29B gene expression, it may be possible that lack of AGP31 + may influence RD29B expression in an alternative route. To see if lack of AGP31 causes an increase in RD29B gene expression in an alternative way than ABA treatment, an experiment designed to sequester and prevent ABA target transcription factor for RD29B with our same BR treatment lines could be carried out. If the 6134-ABA treated lines showed an increase in mean fold gene expression of RD29B comparable to the results shown in graph D, while Ler-ABA treated lines showed a significant decrease in RD29B expression compared to results shown in graph B, then it could be possible to conclude that the loss of AGP31 promotes RD29B gene expression in the presence of ABA molecules via a different molecular route than wildtype ABA lines.

Graphs G and H reflect the roughly the same results as graphs A and B, which is to be as expected. R2-4 lines have the same AGP31+ phenotype as Ler0 lines, and both lines contain the same amount of AGP31 protein level. These results help confirm previous conclusions about ABA having very little significant effect on the transcription of OLE1 gene. Graph H resembles the results in graph B, indicating once again, that RD29B is transcribed at a higher rate in the presence of ABA. It is noted, however, that the mean fold increase for R2-4 lines is about 110, compared to wildtype Ler0 169. This difference in gene expression level may be due to the complementation during reversion of R2-4 to AGP31+ genotype which could have some alternative effects due to reinsertion. Re-testing the same experiment with more BR samples could confirm whether this decrease is due to complementation effects, or random chance due to low sample number.

Further directions of this experiment to investigate the role of ABA and OLE1 expression should include BRs at different times in development. The data obtained in this experiment indicated that there seems to be no significant relationship between OLE1 gene expression and ABA treatment. This may be true of these BR samples (~13 days old) but we may see a correlation between OLE1 and ABA if the BRs were in seed development, or more mature. Seed development confers with a high expression of OLE1, whereas 13 day old seedinglings are known to express lower levels of OLE1. By re-doing this experiment with a larger sample size, as well as BRs at different stages of development, we may be able to see how development, ABA treatment and OLE1 expression relate. Further directions investigating AGP31 involvement in expression of RD29B during ABA exposure may include an experiment designed to sequester and prevent ABA target transcription factor for RD29B. Our same BR treatment lines would be used for this experiment, as previously explained in the results section.