

Investigating the role of CHR9 in LFY dependent transcription in regards to flower development
and the role of epigenetics in induced response to herbivory

Research Thesis

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General Introduction

Many living organisms rely on their DNA to store the information that allows for them to be able to develop, survive, and pass on their genetic information to their eventual offspring. Not all of the genes that DNA codes for are expressed at all the different stages of development in an organism's life. I was able to work on two projects that involved investigating gene expression in the model organism *Arabidopsis thaliana* during my time as an undergraduate. Although these projects differed in their approaches and their ultimate aims, both of them involved investigating the role of different mechanisms involved in the regulation of gene expression. Gene expression is tightly controlled to allow for proper development through the use of transcription factors, activators, repressors and other various methods that can allow for the proper expression pattern of a gene product. In Dr. Rebecca Lamb's laboratory, I investigated the role of CHR9 which is proposed to be a potential cofactor of the LEAFY transcription factor which is a key regulator of reproductive development in *Arabidopsis*. Under Dr. R. Keith Slotkin, I worked on the effect of herbivory on the epigenetic marks of different areas of the genome and the expression of different genes involved in the regulation of these epigenetic marks.

Chapter 1: The role of the chromatin remodeling protein CHR9 in LFY-dependent transcription.

Background

Successful reproduction is crucial for an organism to be able to contribute their genetic information to their population. It allows for a species to preserve segments of their genetic information that allow for their adaptation over time to their environment, leading to a greater chance of survival of said species. In regards to angiosperms, the flower contains the reproductive structures of the plant, the stamens and the carpals. Flowers and their components are created post embryonically by the developing plant. This is able to occur due to the fact that plants have a reservoir of meristematic cells located at both apexes of their body plan. The shoot apical meristem (SAM) is responsible for producing the future cells that will differentiate into the plant organs that are located above the surface of the soil. The root apical meristem (RAM) is responsible for the production of cells that will differentiate into structures below the surface of the soil.

As a result of its importance, reproduction is under tight control by Arabidopsis to insure that it flowers in the appropriate environmental conditions in order to better successfully pass on its genetic information. Growth in Arabidopsis occurs in two specific phases that can be phenotypically observed, the vegetative growth phase and the reproductive growth phase (Fig.1). The vegetative phase involves the development of the rosette leaves laterally around the base of the plant. Then, there is a transition to the reproductive phase of growth which is subdivided into two additional events. First there is an elongation of the bolt from the base of the plant and branches form of this shoot inflorescence as well during this first phase of reproductive growth. Finally, during the second stage of reproductive growth, flowers begin to form off of the shoot

(reviewed in (Siriwardana and Lamb, 2012)). These phenotypic changes during growth gives a researcher the ability to mark the relative transition from vegetative to reproductive growth and time until flower formation.

One of the most important genes involved in regulating reproductive growth in *Arabidopsis* is the *LEAFY* gene. *LEAFY* is a floral meristem identity gene and a key regulator in the formation of the specific whorls of the flower as well as flowering time. In a loss of function *LEAFY* mutant, there is an increase in the number of lateral branches formed off of the shoot before eventual flower formation (Huala and Sussex, 1992). The flower that a loss of function *LEAFY* mutant eventually forms is abnormal in its development and a complete *LEAFY* knockout causes a loss of stamen and carpel formation (Siriwardana and Lamb, 2012). A gain of function *LEAFY* mutation causes a decrease in the number of branches before flower formation and these flowers can even form on the shoot where in a wild type plant there would normally be branches (Weigel and Nilsson, 1995).

The *LEAFY* protein is able to express the genes necessary for the formation of the different whorls of the flower because it encodes a transcription factor (Moyroud et al., 2011; Winter et al., 2011). This transcription factor physically associates with other proteins in a multiprotein transcriptional complex and previous work in the Lamb lab has discovered a potential cofactor of *LEAFY*, *CHR9*, through the use of co-immunoprecipitation techniques (Lamb Laboratory, unpublished data). The *CHR9* protein is a member of the SWI/SNF chromatin remodeler family (Fig 2.). SWI/SNF chromatin remodelers are ATPases that are able to interact with nucleosomes in order to shuffle them in and out of the chromatin structure (Gentry and Hennig, 2014). The movement of these nucleosomes could cause different levels of gene expression of the DNA that was wrapped around them. There are multiple members of the

SWI/SNF chromatin remodeler family that are present in *Arabidopsis thaliana*, 42 in total (Sarnowski et al, 2005). Two SWI/SNF chromatin remodelers, SPLAYED and BRAHMA, have been demonstrated to interact with LFY and are important in activation of different floral organ identify genes involved in flower formation (Miin-Feng et al, 2012). In particular, SPLAYED has been shown to be involved in the regulation of LEAFY expression as a repressor (Wagner and Meyerowitz, 2002). This gives me reason to believe that CHR9, another SWI/SNF chromatin remodeler is involved in reproductive development through its interaction with LEAFY.

During my time in Dr. Lamb's lab I was tasked with the project to investigate CHR9's role in flower development and the transition between reproductive and vegetative growth. Although it has been demonstrated that CHR9 physically interacts with LEAFY (Lamb Laboratory, unpublished data), it is unknown if CHR9 has any role in reproductive development. The hypothesis of the project was that because of the physical interaction between CHR9 and LEAFY, CHR9 will be involved in reproductive development. There were two initial aims of this project. The first one was to characterize CHR9 and the second was to investigate its expression pattern. A third aim was identified during experimentation as observations were made that indicated that CHR9 could have a potential role in the control of germination. Germination experiments were developed and pursued in order to test this aim as well.

Aims of Project

1. Genotypic Characterization of Chr9
2. Investigation of the CHR9 expression pattern
3. Non-inductive germination of *chr9-1* seeds

Genotypic Characterization of CHR9

Materials and Methods

The first aim of my project was to investigate what effect CHR9 had on the flowering phenotype. This was accomplished by using several lines of T-DNA insertion mutants of the CHR9 gene, which caused a loss of function, as well as gain of function CHR9 lines which were under the control of a 35S promoter. These seeds were planted in a growth chamber under long day conditions (16 hours light, 8 hours dark). Next to these gain of function and loss of function lines I also planted *lfy-6* and Col-0 wild type control lines as well. While these plants were developing I was tasked with counting the number of branches that were observed on the shoot inflorescence while another member of my lab, Matt Habina, was tasked with counting the number of rosette leaves that formed during each of these lines development from seed to seed. Through counting rosette leaves I can determine the relative amount of time it takes a genotype to transition from vegetative to reproductive growth. The number of branches will show the relative amount of time a genotype takes to initiate flower formation during reproductive growth. Data from this initial trial can be seen in Table 2.

These preliminary results support my earlier hypothesis that CHR9 is involved in the regulation of flowering time and the transition to reproductive growth. However, it was necessary to examine a larger number of plants to ensure that the phenotypic results that I had observed were not due to chance but were a consistent biologic phenomenon. In the second trial, I planted the same set of *Arabidopsis thaliana* lines again to grow under long day conditions with an addition of a *ddm1* mutant line, a SWI/SNF chromatin remodeler known for its involvement in epigenetic silencing, as another control in my experiment. I also wanted to see if the overexpression or loss of function CHR9 lines would have any changes in their phenotype in

response to a change in day length. Therefore, I planted the same lines under short day conditions as well. During the life time of these plants I counted the number of branches and rosette leaves like was done in the previous experiment. I also counted the number of days before the transition from vegetative to reproductive growth starting with the day that I placed these seeds on soil and the ending day which was when I was able to observe the initial transition from vegetative to reproductive growth. The observed phenotype that denoted the transition had occurred was, when at the base of the rosette leaves, I could see the initial formation of the bolt. At the writing of this thesis, analysis of this data is ongoing and as a result is not listed in the figures section.

I also worked on producing crosses between the *chr9-1* and *lfy-6* plants as well as between the 35S::CHR9 and *lfy-6* plants. I would begin my crosses by isolating the flower of the genotype that I designated as the female of the cross. I would then carefully remove the petals, sepals and immature stamen of these flowers leaving the carpel as the lone component of the flower. I would then rub the stamens from the flower of the designated male genotype onto the aforementioned carpel. A number of the crosses were successful and remain stored to be planted onto soil in the future.

Results

In the preliminary results (Table 2) there is a correlation between the absence and presence of CHR9 and flower development. In the gain of function CHR9 mutants, there were less rosette leaves than Col-0 wild type and they formed fewer branches before flower formation as well. These observations would indicated that 35s::CHR9 lines are able to transition more rapidly from the vegetative phase to the reproductive phase as well as forming their flowers more quickly as well. The exact opposite phenotype can be observed in our loss of function *chr9-1*

mutants. These *chr9-1* mutants produce more rosette leaves and more branches before flower information than our Col-0 wild type control. These observations would indicate that a plant with a loss of CHR9 function would take additional time to transition between vegetative and reproductive growth and to form their flowers. What is interesting about these phenotypes are their correlation between what you would see in an overexpression of the LEAFY gene and the loss of function of the LEAFY gene as discussed in the background section. This observation is also supported by phenotypic observations of my *lfy-6* line correlated with the different CHR9 lines (Table 2).

Discussion

This correlation between my experimental results and previously discovered information about LEAFY would lend credence to my thought that the physical interaction between CHR9 and LEAFY has an effect on LEAFY's ability to initiate reproductive growth. However, this does not prove that CHR9's observed effect is only the result of its interaction with LEAFY. CHR9 could interact with other components of the reproductive development pathway independent of LEAFY as well. This idea is being and will be further explored in the second aim of this project. While my preliminary results appear to be supporting my hypothesis, the number of plants that were observed is too low to make any conclusions. Thus I planted more Arabidopsis lines in order to gather more data. Unfortunately, at this moment I have not been able to properly analyze the data and that analysis is crucial for the future of this project. Also, certain crosses were performed in order to create specific mutant strains in order to better elucidate the relationship between LEAFY and CHR9. The *lfy-6; chr9-1* double mutant phenotype will tell us if these genes effects are additive to one another or are epistatic. The

creation of the *lfy-6; 35S::CHR9* mutant will demonstrate if the overexpression of the CHR9 gene is capable of alleviating the effects of the LFY mutation.

Investigation of the CHR9 Expression Pattern

Materials and Methods

In order to better understand what role CHR9 could potentially have in reproductive development I needed to be able to observe its detailed expression pattern through the creation of a transgene transcriptional reporter line. This transgene contained the 1.1 kb CHR9 promoter driving a GUS coding region fused to a GFP coding region as well. I isolated the CHR9 promoter from genomic DNA through the use of PCR. The primers (Table. 1) used to isolate the CHR9 promoter (CHR9p) amplified the DNA fragment that was between the CHR9 gene and the gene upstream of it ensuring the entire CHR9 promoter was taken. Then, I performed gel electrophoresis in order to confirm the presence of the amplified CHR9p sequence and the positive band was cut out of the gel and was purified in order to enhance our chances of successful cloning reactions. The presence of DNA after gel purification was confirmed using a Nanodrop.

Due to the fact that I want to be able to produce a transgene that is able to have GUS and GFP expression that is under the control of the CHR9p, it was necessary to use directional cloning into the entry vector. The CHR9p sequence was then confirmed in the pENTR/D-TOPO entry vector through extraction of DNA from the plasmid followed by a restriction enzyme double digest to identify positive colonies for the CHR9p. The CHR9p sequence was then placed into a destination vector containing the GUS, GFP, and basta resistant coding regions using an LR reaction. The destination vector, pBGWFS7, was checked for successful integration through the use of a restriction enzyme double digest on purified plasmid DNA. Those colonies that were

confirmed were then sent for sequencing in order to make sure that during the recombination between vectors there were no breaks in the sequence that could affect its biological activity. Sequencing confirmed the colonies that I had sent; therefore I proceeded to transform Col-0 wild type *Arabidopsis* plants using *Agrobacterium* mediated transformation. These transformed plants were then grown in the greenhouse and their seeds were collected and successfully transformed plants will be selected through basta selection.

Discussion

At the writing of this thesis, I have been unable to produce any conclusions about the expression pattern of CHR9 with my transgene. Seeds have been gathered from transformed plants and they will be planted out and selected for with basta herbicide. With these successfully transformed plants, I will be able to perform GUS staining along with observations of GFP to better understand when and where CHR9 is expressed during different stages of development.

Using these transformed plants. I will be able to identify expression of CHR9 at both a tissue specific level as well as a cellular level. Specifically, examining CHR9's expression pattern and correlating it to LEAFY's expression can be helpful in determining whether or not their interaction is associated with reproductive development in *Arabidopsis*. However, if their expression is shown to correlate with one another, this would not confirm that CHR9 acts only with LEAFY to potentially affect reproductive development. There is a possibility that CHR9 interacts with factors or genes that could affect development through a different pathway. In order to determine if CHR9 could interact with factors besides LEAFY, I would like in the future to perform Co-Immunoprecipitation to identify these factors. Then, after identification of these factors, I would characterize their function in a similar method to what I have used to characterize CHR9 function. The examination of the function of potentially interacting genes

would be crucial for the further characterization of CHR9's role in flower formation and the transition from vegetative to reproductive growth.

Noninductive germination of *chr9-1* seeds

Materials and Methods

It was observed earlier during the seed stratification of the *chr9* loss of function lines for aim one that they appeared to undergo early germination even though they were in non-inductive conditions (i.e. placed in a 4 degree freezer and covered with aluminum foil). Thus, I performed several experiments to determine if this is an anomaly or is it a consistent phenotypic effect of this mutation. The first experiment involved plating *chr9-1* seeds on 6 plates and wild type Col-0 seeds on six other plates. These plates were then paired up and labeled "0-5". Plates "1-5" were wrapped in aluminum foil and placed at 4 degrees Celsius while the plate pair labeled "0" was placed into a growth chamber, uncovered. Plates 1-5 were kept covered and in the 4 degrees refrigerator for a predetermined number of days. Plates labeled "1" were taken out after one day, Plates labeled "2" were taken out after two days, and this continued until the last plates were removed. These plates when removed from the refrigerator would be removed from their aluminum foil covering and placed into the growth chamber. Seeds that showed germination were counted for each plate for the duration of the experiment, five days of observation for each plate. This data can then be used to help determine what effect this mutation has on the germination rate of this mutant line. The second trial involved the same methodology but I plated out additional lines which included *ddm1*, *35S::CHR9*, and *lfy-6* to see if any correlation between germination rates can be seen between these different lines. The number of non-germinated seeds for each pair of plates was counted over the duration of seven days. The third experiment involved plating 5 plates with wild type Col-0 seeds and 5 plates with *chr9-1* seeds, covering

them in aluminum foil and placing them into the growth chamber. For 5 days, one pair of wild type and *chr9-1* plates was removed from the growth chamber and the seeds that had germinated were counted. A brand new pair of plates was removed and observed over the duration of five days. This final experiment can be useful in determining if the previously observed phenotypic effect is reliant on day length.

Discussion

Unfortunately, at this moment, I was unable to analyze the raw data from these three separate experiments. If I am able to confirm that these observations are not just an anomaly then I would be interested in determining what are some of the other genes or proteins that CHR9 potentially interacts besides its previously observed interaction with LEAFY. As stated previously in my discussion for aim one of this chapter, I would perform Co-Immunoprecipitation with CHR9 to identify these factors that it physically interacts with. Then, I would go through and identify these factors looking for any that have previously been characterized as being involved in germination.

Conclusion

The preliminary data support my hypothesis that CHR9 is involved in reproductive development. Its effect on flowering time in the mutants observed is similar to what is seen in LEAFY mutants. This further supports the idea that CHR9 and LEAFY interact together to influence gene expression. Unfortunately, data from both continued genotypic characterization and non-inductive germination studies still need to be analyzed. Continued work with my constructed transgene as well as using co-immunoprecipitation techniques will allow for the further characterization of this gene.

Chapter 2: The effect of herbivory on the RdDM pathway and its targets

Background

DNA is the data storage center for a majority of living organisms. These organisms need to be able to access this “hard drive” at the right time and without error. One of the numerous factors that can cause deleterious effects in this stored information are transposable elements. Transposable elements are regions of DNA that are capable of excising themselves from their original location in the genome and inserting themselves into a new location. If excision or insertions of these transposable elements occurs in gene coding regions, promoters or cis regulatory elements there can be problems for the organism’s ability to survive. In *Arabidopsis*, transposable elements are kept in check due to epigenetic silencing along with post-transcriptional silencing. Epigenetics is a general term for methods where gene expression can be altered without changing the genetic code. The *Arabidopsis* plant, through methylation of its transposable elements, is able to silence their expression. The nucleotide base, which is methylated in this epigenetic silencing, is cytosine. There are three different “types” of cytosine methylation, CG, CHG and CHH methylation where H stands for all bases but G. Methyl groups can be placed on to previously unmethylated DNA sequences through a *de novo* methylation mechanism and the methylation status of a sequence can also be maintained throughout replication by maintenance methylation (Law and Jacobson, 2010).

De novo methylation, as well as maintenance methylation, is carried out through the actions of methyltransferases. DRM2 is a methyltransferase in plants that is responsible for the *de novo* methylation of DNA sequences (Pikaard and Schied. 2014). MET1 and CMT3 are methyltransferases that maintain the methylation of the CG and CHG cytosine contexts respectively (Pikaard and Schied. 2014). Epigenetic silencing can occur through two different

pathways that have been identified the RdDM (RNA-directed DNA methylation) (Fig. 3) as well as PTGS (Post Transcriptional Gene Silencing) pathways. In the RdDM pathway, ssRNA is produced through the actions of Pol IV which is subsequently made into dsRNA through the actions of RDR2. DCL3 then interacts with this dsRNA producing 24nt siRNAs that are then loaded into the Argonaute proteins, AGO 4/6/9. These Argonaute proteins are able to deliver their cargo to the proper location due to their ability to interact with RNA transcribed by Pol V. Then, methyltransferases are recruited to allow for the methylation of cytosines causing silencing of gene expression (reviewed in Law and Jacobson, 2010). While the RdDM pathway is involved in the silencing of transposable elements at the transcriptional level, the PTGS pathway is involved in posttranscriptional silencing through an RNAi pathway. In this pathway dsRNA transcripts are transcribed from these transposable elements. This dsRNA is recognized by Dicer proteins which perform similar actions as they do in the RdDM, creating fragmented small noncoding RNAs. Argonaute proteins then interact with these dsRNAs, converting them to ssRNAs. These ssRNAs are able to interact with their complementary sequence allowing for either its destruction or an inhibition of its translation (Alberts et. al, 2008)

Along with the placement of epigenetic marks onto DNA, eukaryotic organisms can also apply similar marks to histone tails creating what is referred to as the “histone code”. These modifications can include phosphorylation, methylation and/or acetylation of different residues in the histone tail. A key modification of the tails, in regards to epigenetic silencing, is methylation. Different histone tail methylation patterns are associated with different methylation and expression patterns of nearby DNA sequences. One primary example of the connection that exists between histone methylation and DNA methylation is in regards to H3K9 methylation. The methylation of H3K9 is carried out by SUVH4/KYP which binds to nearby methylated

DNA as well as to CMT3 which is a DNA methyltransferase (Johnson et al, 2007). This would suggest that the actions of these two proteins encourage the action of the other, correlating the methylation of DNA with H3K9 methylation (Johnson et al, 2007). While methylation of H3K9 is correlated with methylation of DNA, other histone methylation marks are negatively correlated with DNA methylation. One such example is H3K4, where methylated H3K4 causes a change in the loss of methylation status of nearby DNA sequences (Greenberg et al, 2013).

The methylation of cytosine bases through the RdDM pathway is not a permanent mark placed on the DNA sequence but can be removed through the actions of proteins that contain a glycosylase domain. An example of one of these glycosylase proteins is ROS1, which is able to remove a methylated cytosine and replace it with an unmethylated cytosine. Methylation and epigenetic marks responsible for the silencing of heterochromatic regions, like transposable elements, are capable of spreading into euchromatic regions. It has been suggested that these DNA glycosylases are important in maintaining the euchromatic regions of DNA (Law and Jacobson, 2010). It has also been suggested that in response to pathogen attack on *Arabidopsis*, ROS1 demethylation activity is involved in the plant's response allowing for the activation of certain defense genes (Yu et al, 2013). It is currently unknown how these glycosylases are targeting specific loci and this would be an interesting area of research.

One of the most unique characteristic about plants is the fact that they are non-motile. They are unable to flee from extrinsic factors like drought, pathogens or other stresses that motile organisms would be able to avoid. Thus, plants have evolved very complex mechanisms that allow them to respond to these stresses and increase their ability to survive and reproduce. Epigenetic changes in DNA sequences and histones can occur as a result of induced stress to allow for plants to respond to these stresses and potentially lead to a development of a

”resistance” after exposure to stress (Gutzt and Scheid, 2012). Plants are in an ever-evolving arms race versus the various biotic stresses they face in order to survive. One of the plant’s lines of defense against biotic stresses is their ability to recognize different effectors that either a pathogen or an insect exposes them to in their process of attack. This recognition occurs through the activity of endogenous resistance genes or R genes. These R genes, once they recognize biotic stress in the plant, are involved in a myriad of signaling events including changes in methylation in response to pathogen attack (Yu et al. 2012). Plant hormones such as Jasmonic acid have been known to be integral for a plant’s immunity to biotic stress as well (Campos et al, 2014).

Our collaborators in their paper (De Vos et al, 2005) detailed the *Arabidopsis*’s stress response to different biotic attacks. Two of the insects that they had used in their experiments were *M. persicae* and *P. rapae*. *M. persicae* are aphids that use a stylus that is capable of penetrating the plant in order to feed off of sugars residing in the phloem while *P. rapae* is a species of caterpillar that feeds on the plant’s leaves aggressively causing a greater amount of damage in comparison to *M. persicae* (De Vos et al, 2005). This paper showed that not only do hormone levels change in these stressed plants but also demonstrated changes in gene expression (De Vos et al, 2005). I am interested in how and why there are these transcriptional changes in these stressed plants and if components of the epigenetic silencing pathway, RdDM, are affected by these specific biotic stresses. During my investigation, tissue samples were procured from our lab’s collaborators from the Netherlands. There were three different sets of leaf tissue, one that was the control group, one that experienced biotic attack from *M. persicae*, and the other had experienced biotic attack from *P. rapae*. Each set had samples from the time points of 24 hours, 48 hours, and 72 hours after exposure to herbivory attack. Also, I generated an artificially

wounded sample and collected its tissue over three 24 hour intervals. This wounded sample was created by puncturing a leaf five times with a syringe like instrument that mimicked the physical damage the leaf would experience through being attacked by *M. persicae*. While both insects were examined during experimentation the majority of my work was spent investigating the Wounding and *P. rapae* samples.

Aims of project

1. RT-PCR of several plant defense genes, TEs and components of RdDM pathway
2. Bisulfite sequencing of TE elements near upregulated defense genes

RT-PCR of several plant defense genes TEs and components of RdDM pathway

Materials and Methods

As demonstrated by our collaborators, *Arabidopsis thaliana* experiences a change in hormone expression as well as changes in gene expression in response to attack by both pathogens and herbivory attack. I was interested in if the demonstrated changes in gene expression observed are a result of changes of the epigenetic regulation. Our goal was to see if any changes in transposable element and R gene expression in our biotic stressed samples are the results of changes in the RdDM pathway. This was accomplished through the examination of the expression of transposable elements, components of the RdDM pathway and known plant defense genes using qRT-PCR.

The investigation into changes in transposable element expression in my samples would allow me to determine if the previously observed changes in gene expression were a result of changes in epigenetic regulation. Due to the fact that transposable elements in *Arabidopsis* are

under particularly tight epigenetic control, an increase in expression or a decrease in expression is indicative of a change in the overall epigenetic regulation of the plant itself. The transposable elements assayed in this experiment include *Athila6 ENV*, *Atrep3*, *Atrep4* and *Tag2* (Fig. 4). If changes in transposable elements were observed it is necessary to determine if the RdDM pathway is affected in a way that could explain this change in expression.

The RdDM pathway is composed of several different genes, some of which are explained in the background of this chapter (Fig. 3). The categories of RdDM involved genes that were assayed through qRT-PCR include methyltransferases (Fig. 7), demethylases (Fig. 8), polymerases (Fig. 6), Dicer and Argonaute (Fig. 5) genes. Changes in the expression of these genes can result in either a complete loss of the RdDM pathway or a reduction in its ability to contribute to epigenetic silencing. As discussed in the background, DNA demethylation has been shown to occur in response to pathogen attack. Our theory is that plants, through the regulation of different RdDM pathway components, relieve epigenetic silencing in response to this herbivory induced stress. This relief of epigenetic silencing would lead to a loss of methylation and expression of previously RdDM silenced genes like transposable elements. Those transposable elements can cause silencing by being located near genes, inside their promoter or cis-regulatory elements as the result of their own silenced state.

Loss of methylation in these newly active transposable elements due to changes in the RdDM pathway can result in activation of nearby genes. Genes of particular interest in investigating to see if there are changes in expression as a result of this loss of methylation are the defense genes, the R genes. The methylation status of these transposable elements near the defense genes, which experience a change in expression, will be investigated in aim two of my project. This change in methylation, if a decrease, would correlate with an increase in expression

of the gene while an increase in methylation in nearby transposable elements would correlate with a decrease in expression.

The change in expression of these different transposable elements and genes were investigated through the use of qRT-PCR. In preparation for qRT-PCR, RNA was extracted from the necessary plant tissues through a Trizol life technologies extraction protocol or was already currently available due to previous experiments. This RNA was then treated with DNase in order to remove any further traces of DNA present in my samples. This allowed for me to create cDNA of, specifically, mRNA in my samples through the use of an oligo-dT primer. These cDNA samples were then placed into a 96-well plate along with primers specific for the gene in question and this plate was run through a qPCR machine. Several new aliquots of cDNA were required to be produced and for each new produced cDNA aliquot, the At1g08200 gene was used as a housekeeping gene with which I could compare the expression of my target genes. The relative expressions of both wounding and *P. Rapae* target genes were compared to the wild type control. The determination of the significance between expression levels of the samples was determined through the use of a two-tailed unpaired t-test with Welch's correction using the computer program Prism. My hypothesis was that in response to biotic stress, Arabidopsis would down regulate certain components of its RdDM pathway sequence to allow for a relief of the epigenetic silencing of transposable elements. This increased expression of transposable elements would be positively correlated with an increase in expression of nearby R genes.

Results

The expression of both *Tag2* and *Athila6* ENV were investigated to determine if mechanical stress, wounding, or biotic stress, *P. rapae*, is able to affect in anyway the epigenetic silencing mechanism that would normally keep transposable elements silenced. For these

targeted transposable elements, the *Athila6* ENV change in expression was insignificant but *Tag2* did show a significant increase in expression in comparison to the wild type sample (Fig. 4). Our hypothesis is that any change in transposable element expression is the result of changes in expression of different RdDM components. Several categories of genes involved in epigenetic silencing were investigated through qRT-PCR including Dicer, Argonaute, Methyltransferase, Polymerase, and Demethylase genes. The polymerases, Pol IV and Pol V, along with dicers, DCL3 and DCL4 did not show a significant change in expression. Unlike the Dicer and polymerase genes, there is a change seen in Argonaute proteins with AGO4 in *P. rapae* showing a decrease in expression compared to the control (Fig. 5). Out of the methyltransferases that were assayed, MET1 did show a significant decrease in expression in the *P. rapae* sample (Fig. 7). Along with methyltransferases, demethylases were examined as well to investigate if an increase in transposable element expression is the result of an active removal of cytosine methyl groups. DML3 is a DNA glycosylase which shows an increase in expression in the wounded sample but not the *P. rapae* infested sample (Fig. 8). Along with DML3, which is a DNA demethylase, histone demethylases were examined as well. As mentioned in the background, the methylation status of histones and DNA are at times correlated with one another, whether a positive correlation or a negative correlation. JM14 and IBM1, both genes involved in histone demethylation, show a decrease in expression in the *P. Rapae* affected sample (Fig. 8).

In response to biotic stress, plants use different signaling events to induce the expression of different resistance genes. Four different R genes were selected for investigation to see if their expression levels change as a result of the stress. Only one of the assayed R genes, AT1G63880, was shown to significantly increase expression in the *P. Rapae* sample (Fig. 9). Two

transposable elements located near a known R gene, PR1, *Atrep3* and *Atrep4* were investigated through qRT-PCR and changes in their expression were insignificant.

Discussion

The expression of *Tag2* and *Athila6* were investigated to determine the effect of the *P. rapae* biotic stress, if any, on the epigenetic silencing pathways in Arabidopsis. *Athila6* expression is known to be affected by changes the epigenetic silencing in Arabidopsis with a *ddm1* mutant, increasing its expression dramatically. In both the Wounding and the *P. rapae* sample, the differences between them and the control in regards to the *Athila6* ENV are insignificant. While the expression of DDM1 in both wounded and *P. rapae* stressed plants is significantly decreased (Fig. 7) it does not seem to cause a significant change in *Athila6* expression. However, unlike *Athila6* ENV, *Tag2* expression in *P. rapae* stressed plants does show a significant increase. This biotic stress is capable of causing changes in epigenetic silencing but not to the extent of a *ddm1* knockout mutant. This would suggest that the RdDM pathway is likely still in effect but not as effective in its silencing ability in wild type.

Several different genes involved in the RdDM pathway were investigated to see if they had a change of expression that could potentially explain the increase in *Tag2* expression. One such component, AGO4 experienced a decrease in expression in the *P. rapae* sample. This decrease in expression may create a situation where the fragmented dsRNA created through Dicer activity will be unable to be targeted by AGO proteins at the same rate as before. AGO6 did not show a significant change in expression and, unfortunately, AGO9 data could not be examined due to an unsuccessful qPCR reaction. In future experiments, I plan on repeating qPCR with AGO9 as a target gene to see if it decreases in expression like AGO4. The categories

that had the most genes affected by these stresses were the methyltransferases as well as the demethylases, both DNA and histone.

While expression change in the methyltransferases CMT2 and DRM2 samples was not significant, the MET1 *P. rapae* sample did show a significant decrease in expression (Fig. 7). This data, combined with DDM1 decrease in expression, would suggest that the change in epigenetic silencing as a result of these biotic stresses not only affects the *de novo* methylation of sequences but the maintenance of a sequence's methylation status as well. Since MET1 is involved in the methylation of CG contexts it would be of interest in future experiments to determine the extent of the loss of CG methylation in these samples through whole-genome Bisulfite sequencing. In further support of the idea that changes in epigenetic silencing in these stressed plants are the result of changes in methylation is the observed expression levels of both DNA and histone demethylases.

An increase of DNA demethylase DML3, which was seen in the Wounded sample, could result in a removal of methyl groups through an active mechanism. Due to the fact DML3 shows an increase in expression only in the wounded plants it maybe a response that is taken in a situation where only mechanical stress is applied and no effectors from a biotic stress occurs. Also, this artificial wounding, while enlightening on ways mechanical stress can affect gene expression, is more representative of a *M. persicae* stress on the plant rather than a *P. rapae*. To identify gene responses that are the result of mechanical stress and/or actual *P. rapae* biotic stress an experimental system that can replicate the caterpillar's mechanical stress needs to be deployed. One of the more interesting changes of expression that was found was the decrease in the expression of JMJ14 and IBM1. JMJ14 has a known H3K4 demethylase activity (Greenberg et al, 2013) and IBM1 has a known H3K9 demethylase activity (Pikaard and Schied, 2014). An

increase of methylation of different histones can be correlated with either an increase in methylation or decrease in methylation of the DNA sequence. H3K9 methylation is, as discussed in the introduction, positively correlated with DNA methylation while H3K4 methylation is negatively correlated. In future experiments I believe it would be an area of interest to correlate these different histone marks with specific R genes in the genome. If R genes are preferentially located around H3K4, I would propose that the down regulation of these two demethylases serves to enhance expression of these R genes while silencing loci that would hamper resistance by commanding some of the plant's finite resources.

The *Atrep3* and *Atrep4* transposable elements showed no change in expression. This however is not a surprising due to the fact that PR1 expression was examined in our collaborators paper and was found not to change in expression in response to *P. rapae* biotic stress (De Vos et al, 2005). This data shows that at R genes not involved in the plant's defense response retain the same expression level of the nearby transposable elements in both the control and *P. rapae* sample. The identification of R genes through RT-PCR which are affected by these specific attacks and the investigation of transposable elements nearby would show if there is a correlation between R gene expression and nearby transposable elements. One such R gene that was affected by *P. rapae* was AT1G63880 which increased in expression. Whether or not this increase is the result of changes in epigenetic silencing needs to be tested in future experiments. Using bisulfite sequencing of nearby transposable elements to AT1G63880 will show if there is a significant change in methylation correlated with increased expression. Also, as discussed in the previous paragraph, I would like to examine if this loci preferentially associates with H3K4.

Overall, while transposable element expression is not at the elevated levels that are seen in an RdDM pathway knockout, like *Athila6* in *ddm1*, expression does increase in in *Tag2*.

Through the examination of different RdDM components, AGO4, MET1 and DDM1 were found out to decrease in expression in the *P. rapae* sample as well as the wounded sample for DDM1. Not only would these changes potentially inhibit the targeting of normally silenced genes by the RdDM pathway, it could also decrease the ability to place methyl groups on cytosines and maintain them. An interesting result from our experiments is a decrease in the expression of two histone demethylases, JMJ14 and IBM1. This change of expression could explain how the reduction of RdDM pathway during stress can target specific loci for expression and at the same time decreasing the expression of other loci.

Bisulfite sequencing of different TE elements near upregulated genes

Materials and Methods

Transposable elements are commonly found near genes or inside their promoters. The methylation of these transposable elements can at times “bleed over” and cause silencing of nearby genes. Genes that experienced an increase of expression through RT-PCR were examined for nearby transposable elements. If the RdDM pathway was involved in this stress induced response, we would observe a change in the methylation patterns nearby these upregulated genes. This potential change in methylation pattern was investigated using bisulfite conversion, which converts the methylated cytosines in a DNA sequence to uracil, which can subsequently be detected through sequencing. This technique allows for the identification of the amount of cytosine methylation present in our samples as well as the context of this methylation, if it is CG, CHG, or CHH. I performed bisulfite sequencing on leaf tissue that included the *P. rapae* affected samples as well as the artificially wounded plant sample. This allowed me to distinguish if methylation pattern differences are the result of mechanical stress, reminiscent of *M. persicae*, or are there responses that are dependent on potential effectors that the insect introduces to the plant

during its feeding. Leaf samples had their genomic DNA extracted and then treated with RNase. The resulting RNA free genomic DNA underwent bisulfite conversion and bisulfite PCR for the ENV and LTR regions of the *Athila6* transposable element. *Athila6* is a retrotransposon located near the centrosome and, as a result, is heterochromatic in nature. *Athila6* was selected because if there were changes in the RdDM pathway that resulted in its expression in qRT-PCR this would likely be accompanied by a loss of methylation. These regions were cloned into TOP10 cells and were checked for successful transformation through colony PCR. Successful transformants for the *Athila6* ENV region was sent to sequencing and its sequence was compared across sample types (Fig. 10).

During the investigation of gene expression changes through qRT-PCR a gene of interest, LAZ5 was discovered to have shown a potential increase in expression (Fig. 9). LAZ5 is an R gene in *Arabidopsis* that is involved in defense against bacterial pathogens. Specifically, LAZ5 is involved in the process of directed cell death as an immune response with an increase in expression of LAZ5 resulting in said response (Palma et al, 2010). LAZ5 is near the transposable elements *Vandal22* and *Atrep10D*. I made bisulfite converted DNA of the Control and *P. rapae* samples and used those bisulfite DNA samples and amplified *Vandal22* and *Atrep10D* using bisulfite PCR. The resulting PCR mixtures were purified through gel extraction and cloned into TOP10 cells. Unfortunately, I was unable to progress this part of my experiment to the sequencing stage.

Results

The methylation of each cytosine context in then control wounded, and *P. rapae* samples were examined and then compared (Fig. 10). The cytosine contexts present in the *Athila6* ENV region for all sample types had error bars, which overlapped one another. These results would

indicate that there is no difference in methylation between the samples. This is not a surprise after determining *Athila6* ENV expression in qRT-PCR is not significantly different between the *P. rapae*, Wounding and Control samples.

Discussion

As discussed in aim one's discussion, *Tag2*, unlike *Athila6*, did show a significant expression. Thus it is not surprising that there was no change in the methylation of *Athila6* because there was no change in expression in that transposable element. Investigating different regions of *Tag2* regions would provide data that would better elucidate if the loss of MET1 expression results in a loss of methylation at the CG context. Also, after analysis of my qPCR data, I realized that although LAZ5 appears to have a higher expression level in *P. rapae* samples, it is not significant. While experiments to examine the methylation of *Vandal22* and *Atrep10D* are underway I believe it would be better to identify transposable elements near AT1G63880. AT1G63880 did show a significant increase in expression in *P. rapae* affected samples. Bisulfite sequencing of nearby transposable elements could help determine whether this increase of expression is due to changes in methylation of nearby heterochromatic regions.

Conclusion

A plant's ability to respond to its environment is crucial for its survival. One stress that requires a plant to begin a cascade of signaling events is a response to a biotic stress like pathogens and insects. During my time in Dr. Slotkin's lab, German Martinez-Arias and I investigated the effect of *P. rapae* biotic stress on Arabidopsis and the potential role of the RdDM pathway in the plant's response. The investigation into transposable element expression using qRT-PCR showed an increase in expression of *Tag2*. This supported the hypothesis that *P. rapae* biotic stress is capable of influencing the epigenetic silencing of transposable elements.

The silencing of these transposable elements is through the RdDM pathway and our qRT-PCR data demonstrates that there is a decrease in the expression of AGO4, MET1 and DDM1. This decrease in expression may be the cause behind the increase in *Tag2* expression and a decrease in the RdDM pathway's activity. Bisulfite sequencing of *Tag2* will be a focus in the future to show that its increase in expression is the result of a loss of methylation.

A potential method for the selective expression of certain loci in response to *P. rapae* biotic stress is seen through the observed decrease in JMJ14 and IBM1 expression. These histone demethylases interact with different histone marks, JMJ14 with H3K4 and IBM1 with H3K9. Methylated H3K4 is negatively correlated with DNA methylation (Greenberg et al, 2013) and H3K9 methylation is positively correlated with DNA methylation (Johnson et al, 2007). This decrease in expression of these different demethylases could create euchromatic and heterochromatic regions in the genome. Through this method a plant could selectively express the necessary genes to respond to a stress event while also shutting off processes that are at the moment unnecessary. Using chromatin immunoprecipitation techniques in future experiments can help determine if genes involved in plant defense are correlated with H3K4.

While the RdDM pathway and transposable elements are affected by this biotic stress, more experiments are necessary to link this data with the increased expression of AT1G63880. Bisulfite sequencing of transposable elements nearby or in AT1G63880 will allow us to correlate a decrease in methylation of the DNA sequence with an increase in expression of this R gene. This would lend credence to the hypothesis that the relief of the RdDM pathway is a method for Arabidopsis to regulate gene expression in response to *P. rapae* biotic stress. Identification of more R genes that experience a change in expression and performing further bisulfite sequencing can link changes in the RdDM pathway with the plants response to the *P. rapae* biotic stress.

Acknowledgements

I would like to thank both Dr. Rebecca Lamb and Dr. R. Keith Slotkin. It was through my interactions with both of these professors that fostered my love for science. It was an honor and privilege to have worked with both of these professors who are not only successful researchers, but they also take it upon themselves to be excellent mentors to those students in their lab as well. I had the opportunity to work with several students during my time as an undergraduate researcher. During my time in Dr. Rebecca Lamb's lab I worked with Matthew Habina who was involved in counting rosette leaves for the different genotypes seen in Table 1. In Dr. Slotkin's lab I was able to interact with all of the graduate students in the lab, Andrea McCue, Dalen Fultz, Saivageethi Nuthikattu, Kaushik Panda, and Meredith Sigman. Through these interactions I was able to develop myself scientifically and I am ever grateful for their help. I know I will be able to carry what I have learned in both of these labs to my future work. During my work in Dr. Slotkin's lab I researched with German Martinez-Arias, a post-doctoral researcher. He helped me out with any questions or concerns I had about our project. German was also involved in experiments as well and was involved in performing qRT-PCR with me. Finally, I would also like to thank my family for being by my side during my years as an undergraduate. They encouraged me and at the very least pretended to be interested when I talked to them about what I was doing in the lab.

Figures and Tables

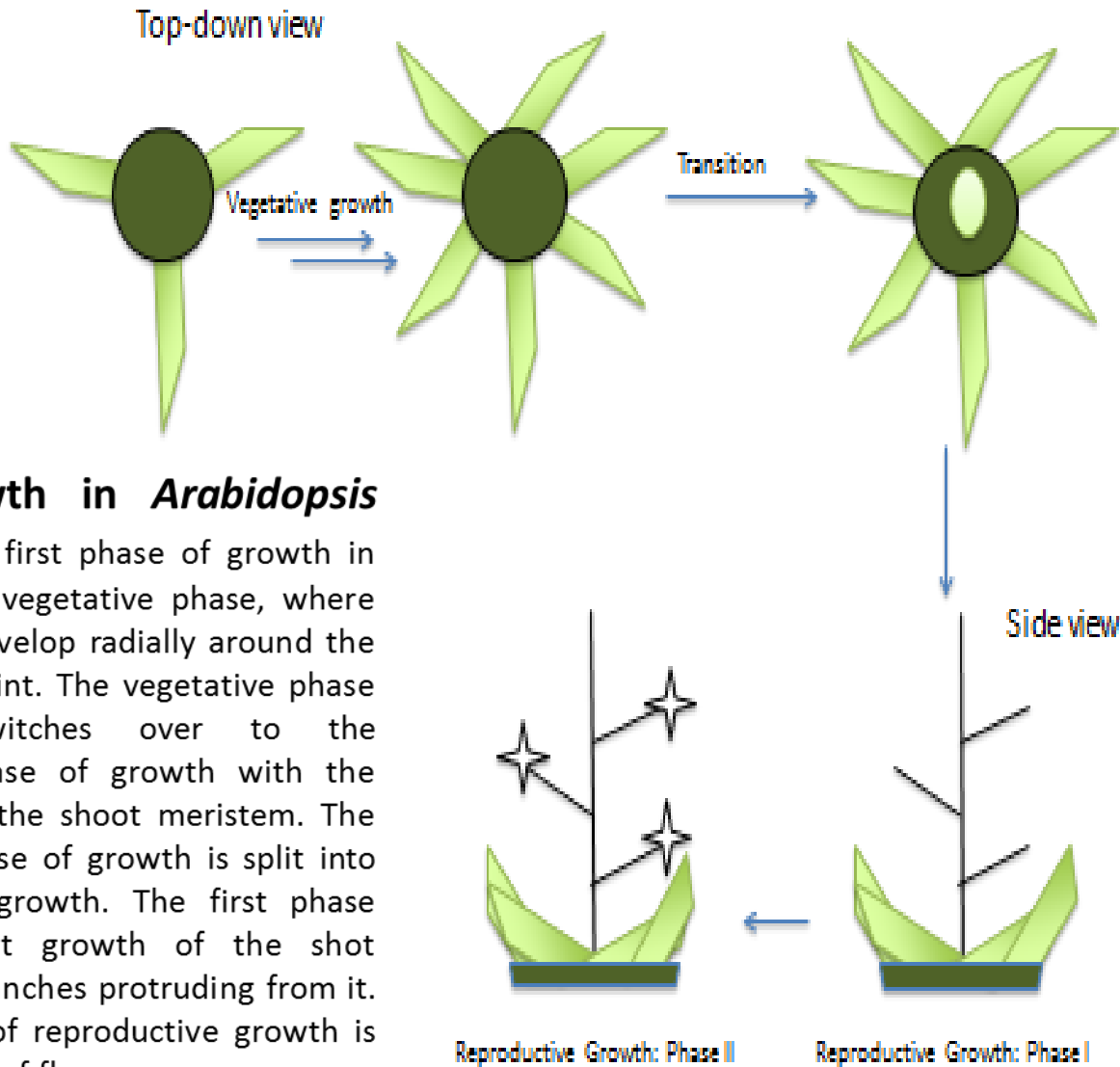


Fig 1. Growth in *Arabidopsis*

***thaliana*:** The first phase of growth in *Arabidopsis*, the vegetative phase, where rosette leaves develop radially around the plant's center point. The vegetative phase of growth switches over to the reproductive phase of growth with the development of the shoot meristem. The reproductive phase of growth is split into two phases of growth. The first phase involves the out growth of the shoot meristem and branches protruding from it. The final phase of reproductive growth is the development of flowers.

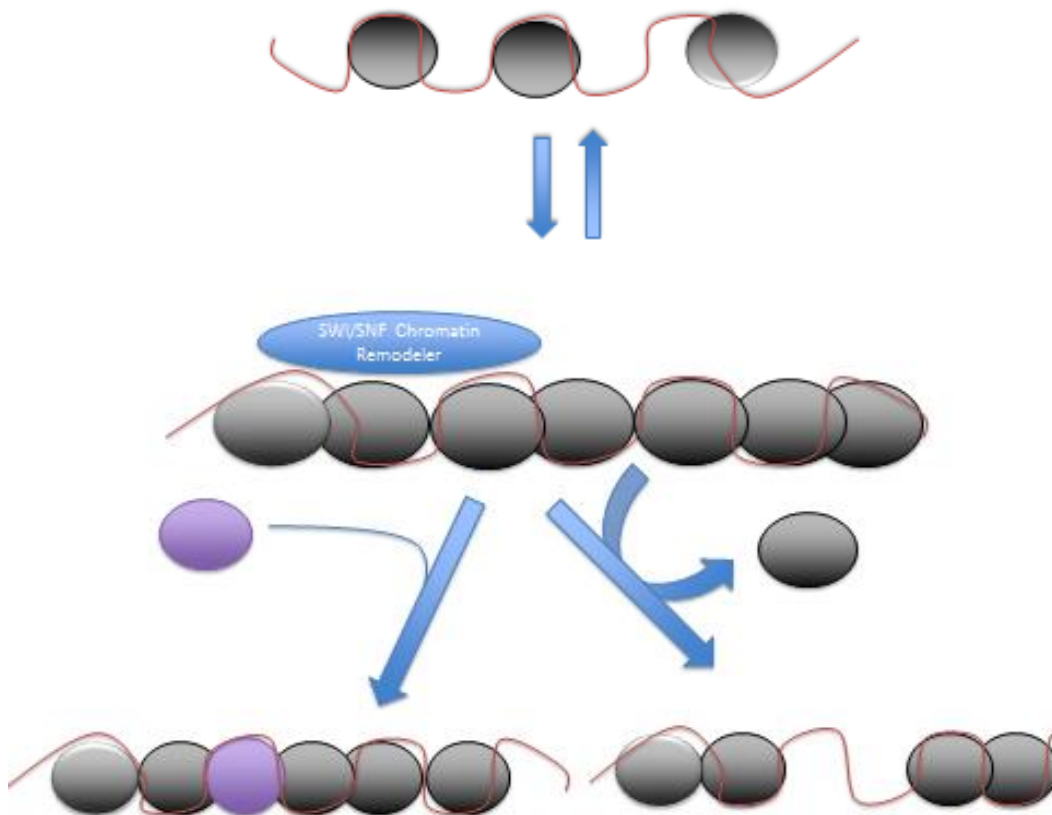


Fig 2. SWI/SNF chromatin remodeler activity: SWI/SNF chromatin remodelers are ATPases that are able to modify the chromatin structure through the hydrolysis of ATP. This action can allow for the shuffling in and out of different histone variants as well as the removal of different histone variants as well.

Primer name	Sequence of primer
CHR9PF2	5'-CACCAATCATTGG.....CAATTATTGAGC-3'
CHR9PR	5'-CGCTAATCGATCGCTTGCGAG-3'

Table 1. Primer table for CHR9p: Both the forward and reverse primers used to isolate the CHR9 promoter are in this table

GENOTYPE	N ^a	NUMBER OF ROSETTE LEAVE AT FLOWER ^b	NUMBER OF BRANCHES BEFORE FLOWER FORMATION
Col-0 WT	15	5.7 (0.5) ^c	3.5 (0.7)
<i>lfy-1</i>	10	6.1 (0.3)	12.1 (0.9)
<i>Chr9-1</i>	12	9.8 (0.2)	5.6 (0.8)
<i>35S::CHR9</i>			
Line 1	10	4.3 (0.4)	2.9 (0.7)
Line 2	14	5.1 (0.6)	2.7 (0.3)
Line 3	10	4.5 (0.3)	N.D.

Table 2. Rosette and Branch number ^A Number of plants analyzed. ^B Plant grown under long day conditions (16 hours dark 8 hours dark). ^C Standard Deviation.

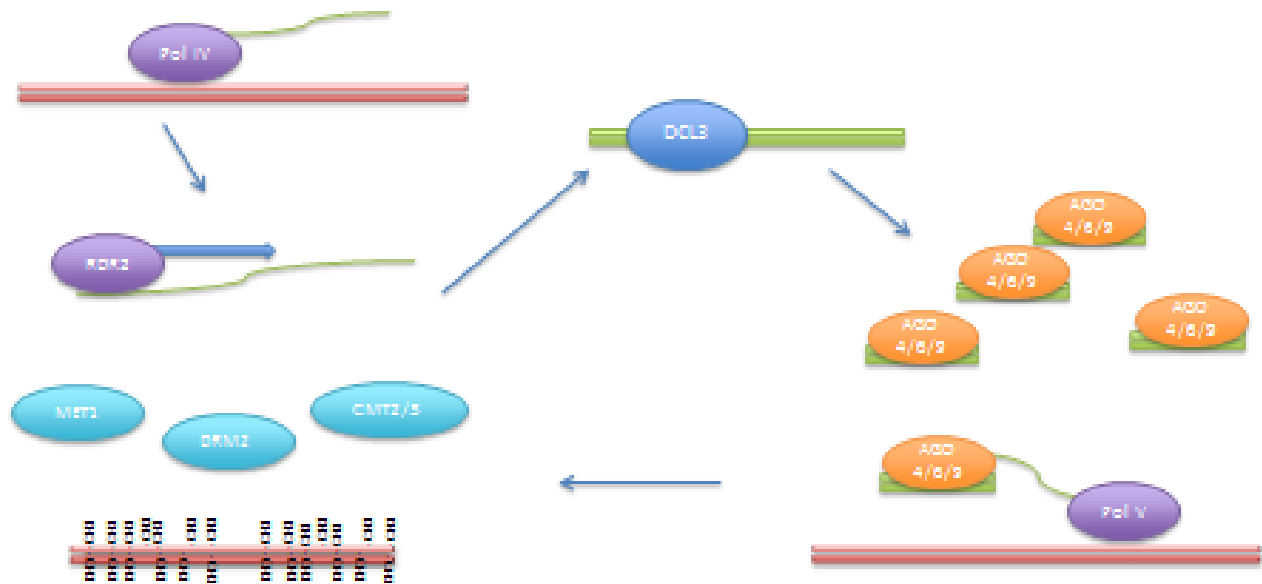


Fig 3. The RdDM pathway and it's components:

When a transposable element is active, polymerase IV produces a ssRNA of the active transposable element. RDR2 creates a dsRNA strand from said ssRNA. This dsRNA is cut up into fragments. These small RNAs are recognized by the Argonaute proteins which then are targeted to these TEs through interactions with a scaffold RNA produced through polymerase V activity. This recruits DRM2 for de novo methylation of the sequence followed by MET1 and CMT2/3 activity as well.

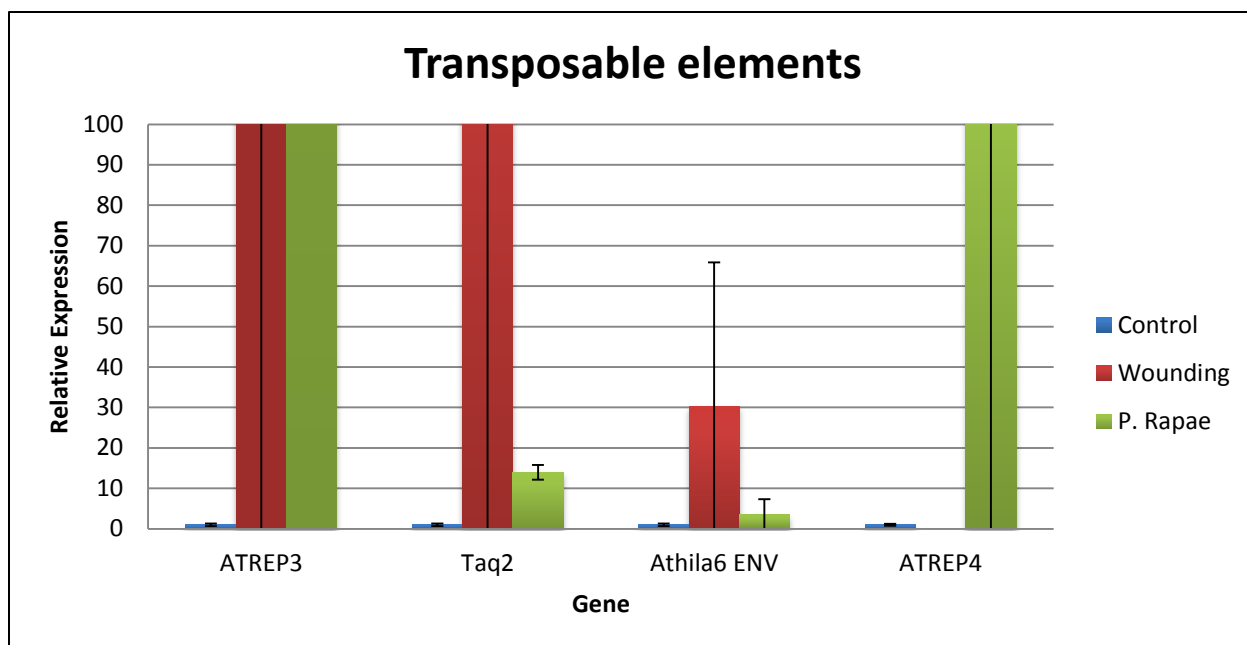
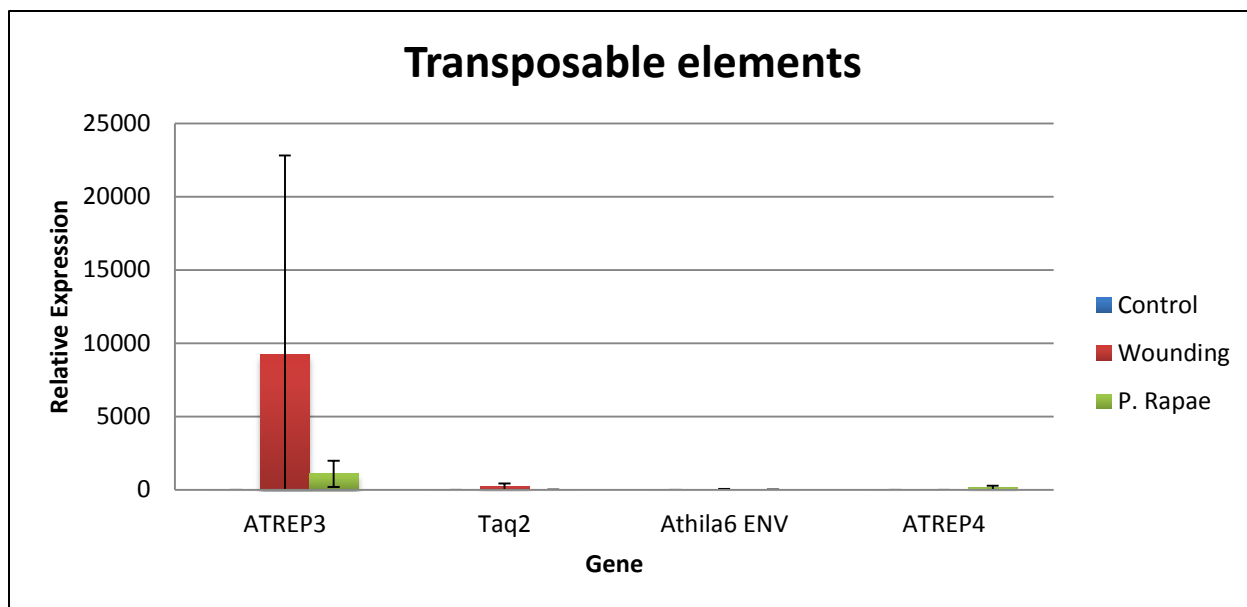


Fig 4. qRT-PCR expression for transposable elements: Relative expression data of transposable elements in wounded and *P. rapae* samples in comparison to the control sample. Both of these graphs are of the same data but in different scales. *Tag2* shows a significant difference between its *P. rapae* sample and the control.

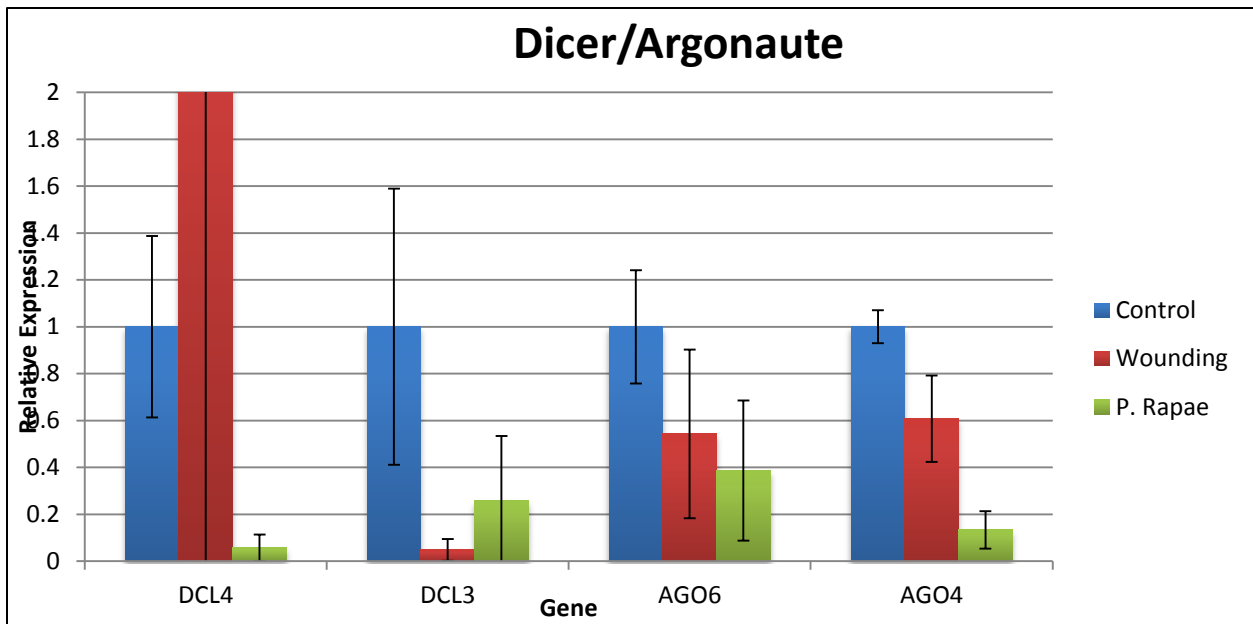
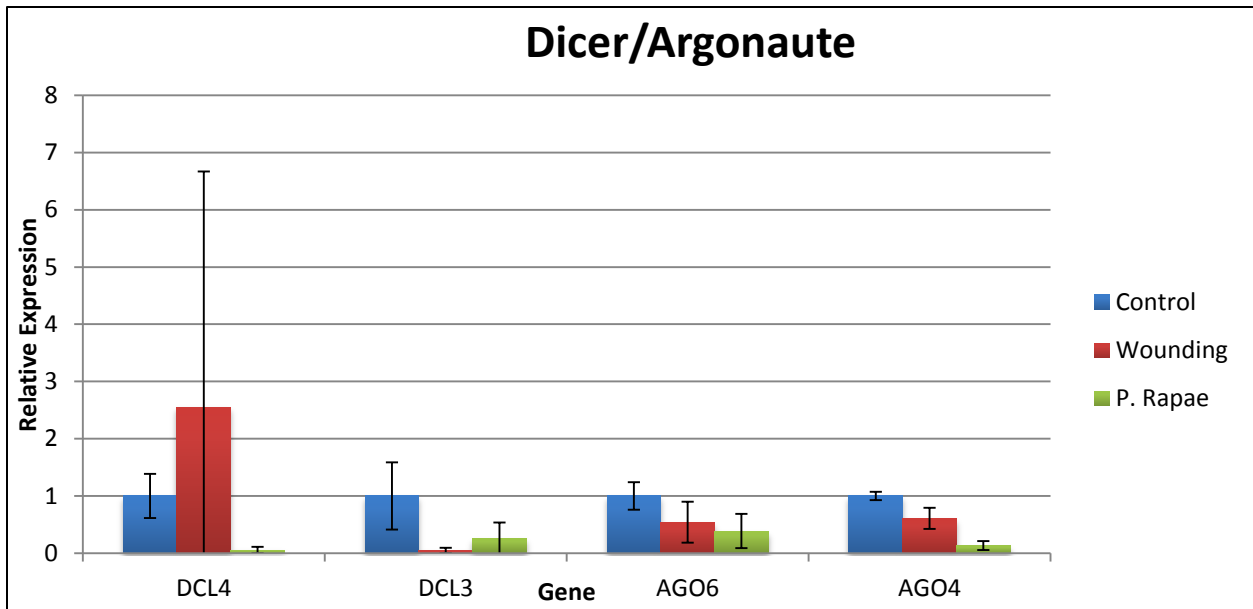


Fig 5. qRT-PCR data for Dicer and Argonaute genes: Relative expression data of Dicer and Argonaute genes in wounded and *P. rapae* samples in comparison to the control sample. Both graphs are of the same data but in different scales. AGO4 shows a significant difference of expression between its *P. rapae* sample and the control.

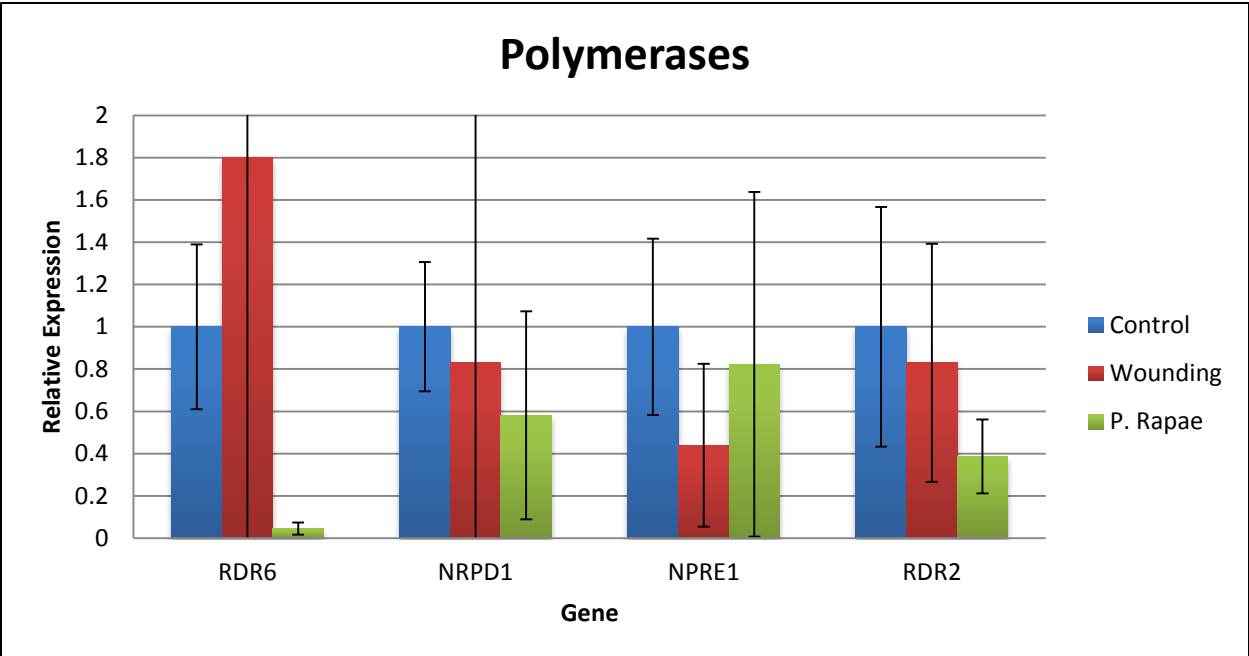
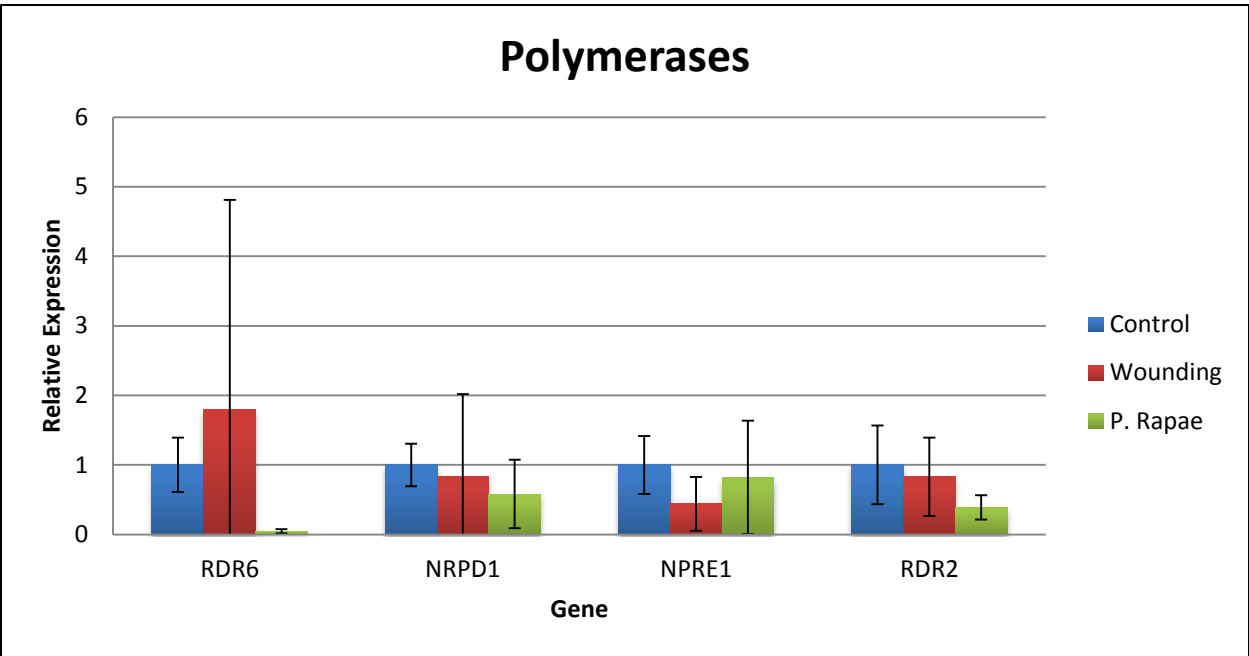


Fig 6. qRT-PCR data of polymerases: Relative expression data of polymerases in wounded and *P. rapae* samples in comparison to the control sample. Both graphs are of the same data but in different scales .

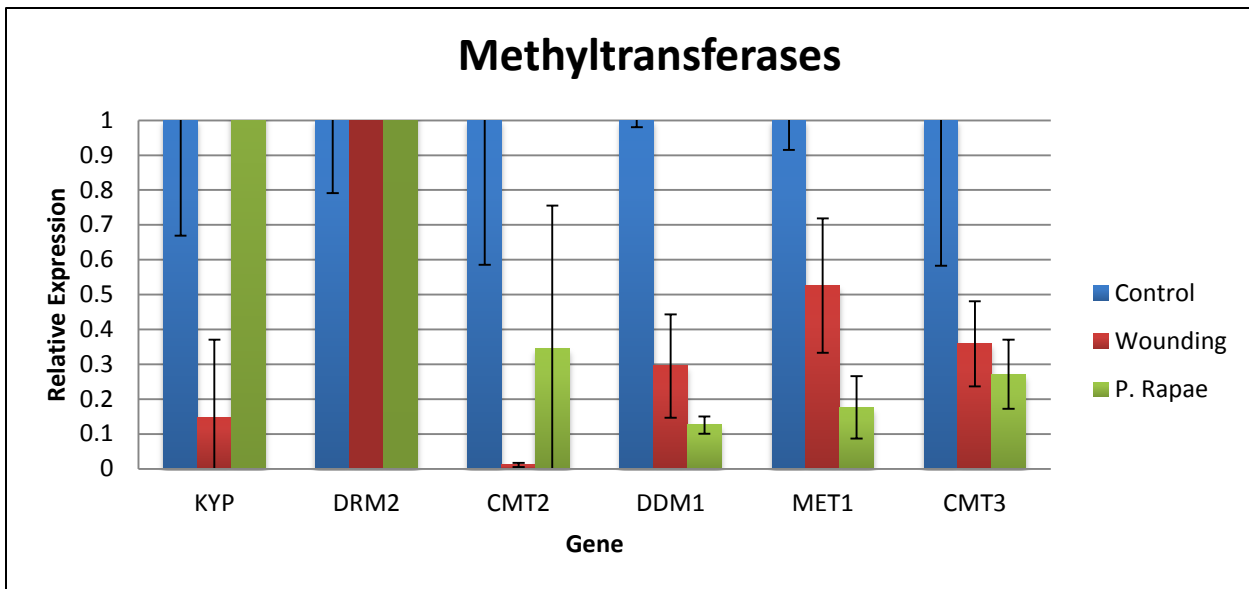
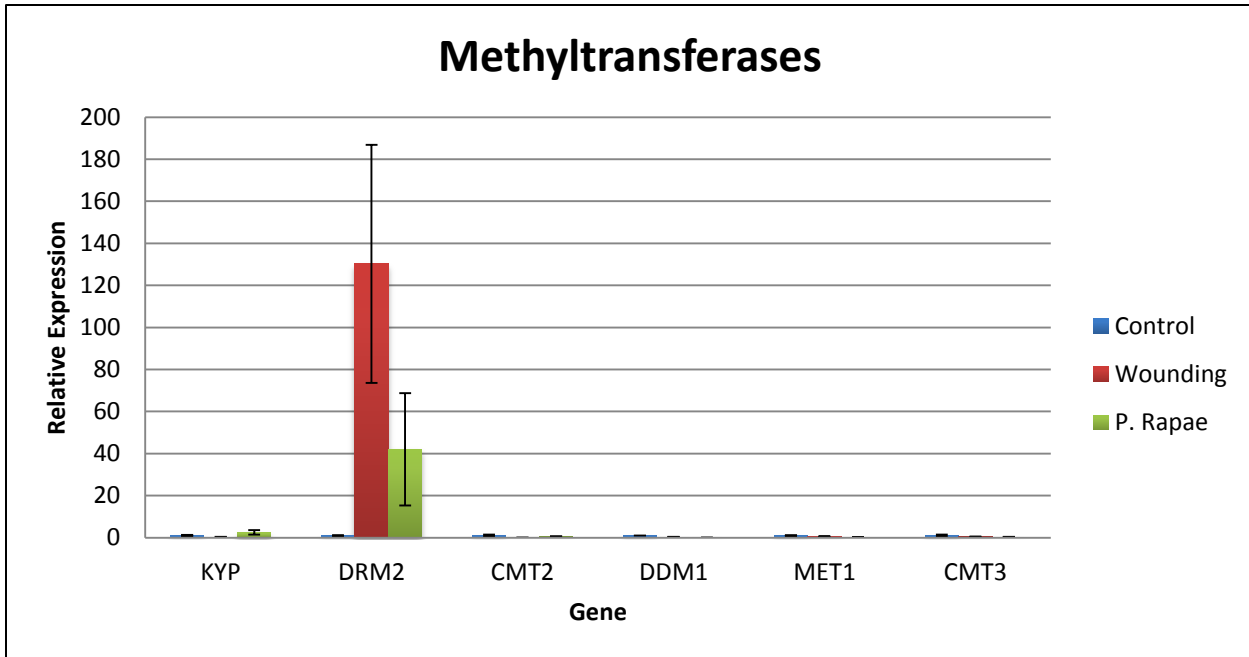


Fig 7. qRT-PCR data of Methyltransferases: Relative expression data of methyltransferases in wounded and *P. rapae* samples in comparison to the control sample. Both graphs are of the same data but in different scales. DDM1 shows a significant change in expression in comparison between the control and both the wounded and *P. rapae* samples. MET1 shows a significant change in expression between *P. rapae* and the control.

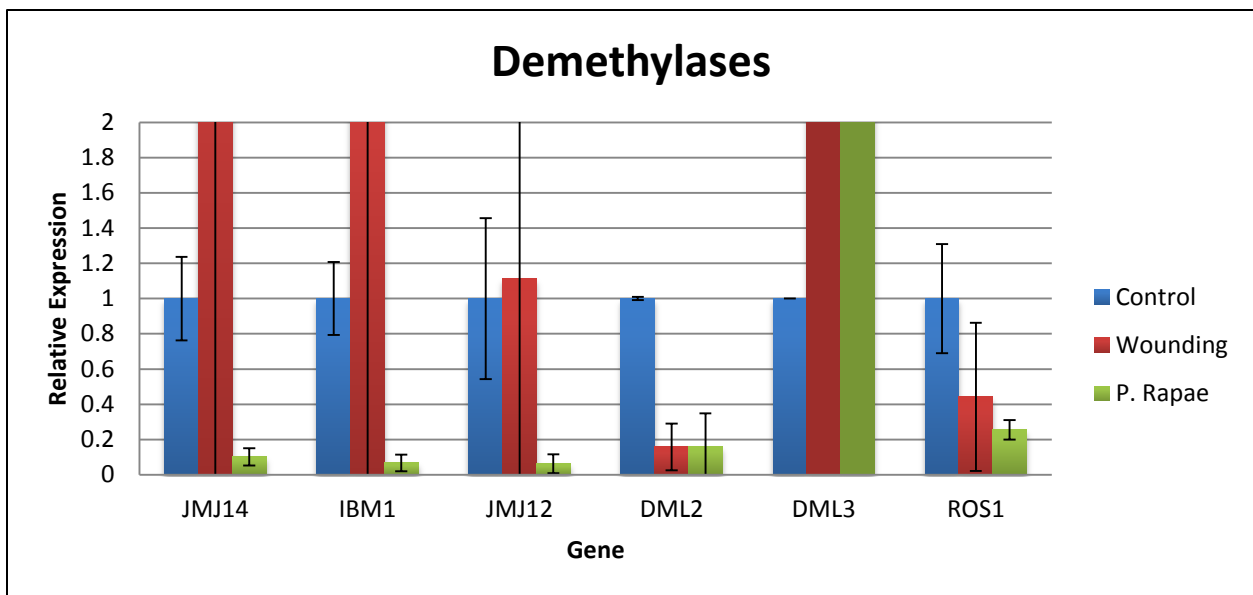
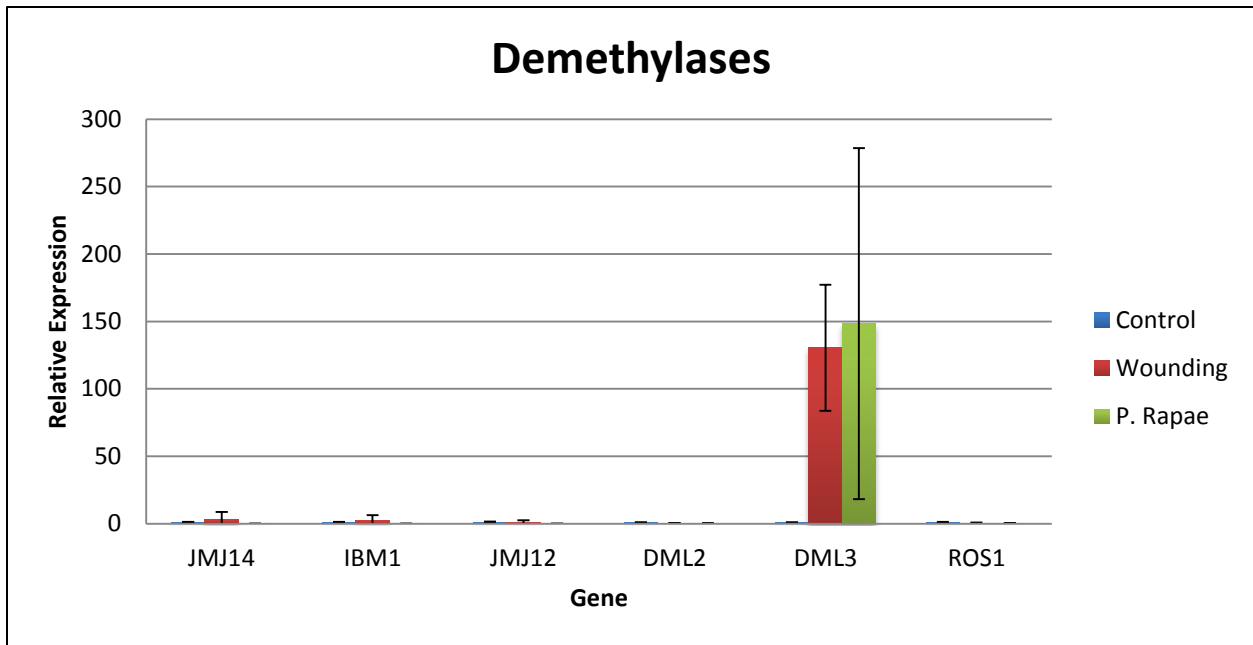


Fig 8 qRT-PCR data of Demethylases: Relative expression data of demethylases in wounded and *P. rapae* samples in comparison to the control sample. Both graphs are of the same data but in different scales. JMJ14 and IBM1 show a significant change in expression between the *P. rapae* sample and the control. DML3 shows a significant change in expression between the control and the wounded sample.

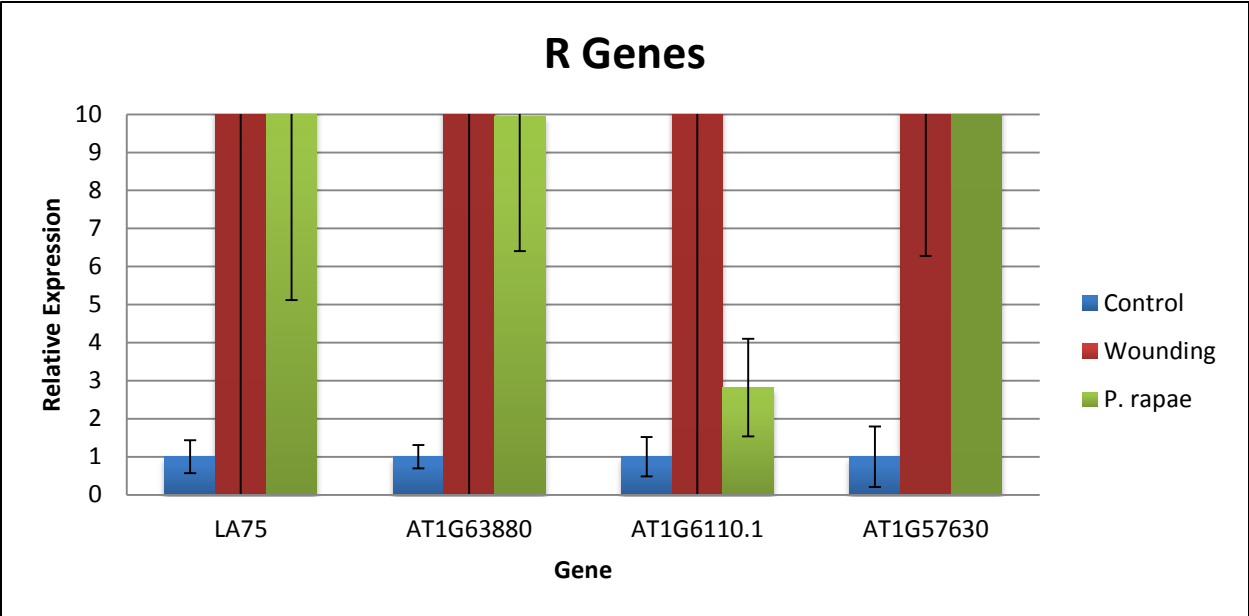
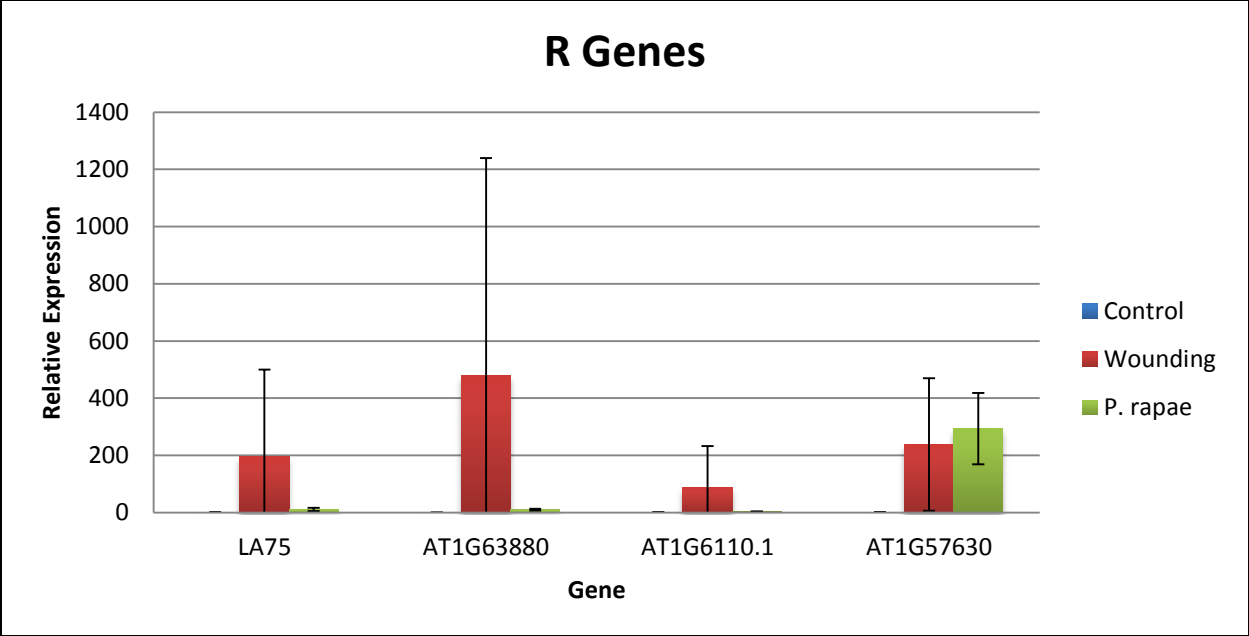


Fig 9. qRT-PCR data of R genes: Relative expression data of R genes in wounded and *P. rapae* samples in comparison to the control sample. Both graphs are the same data but in different scales. AT1G63880 shows a significant difference between the *P. rapae* and control sample.

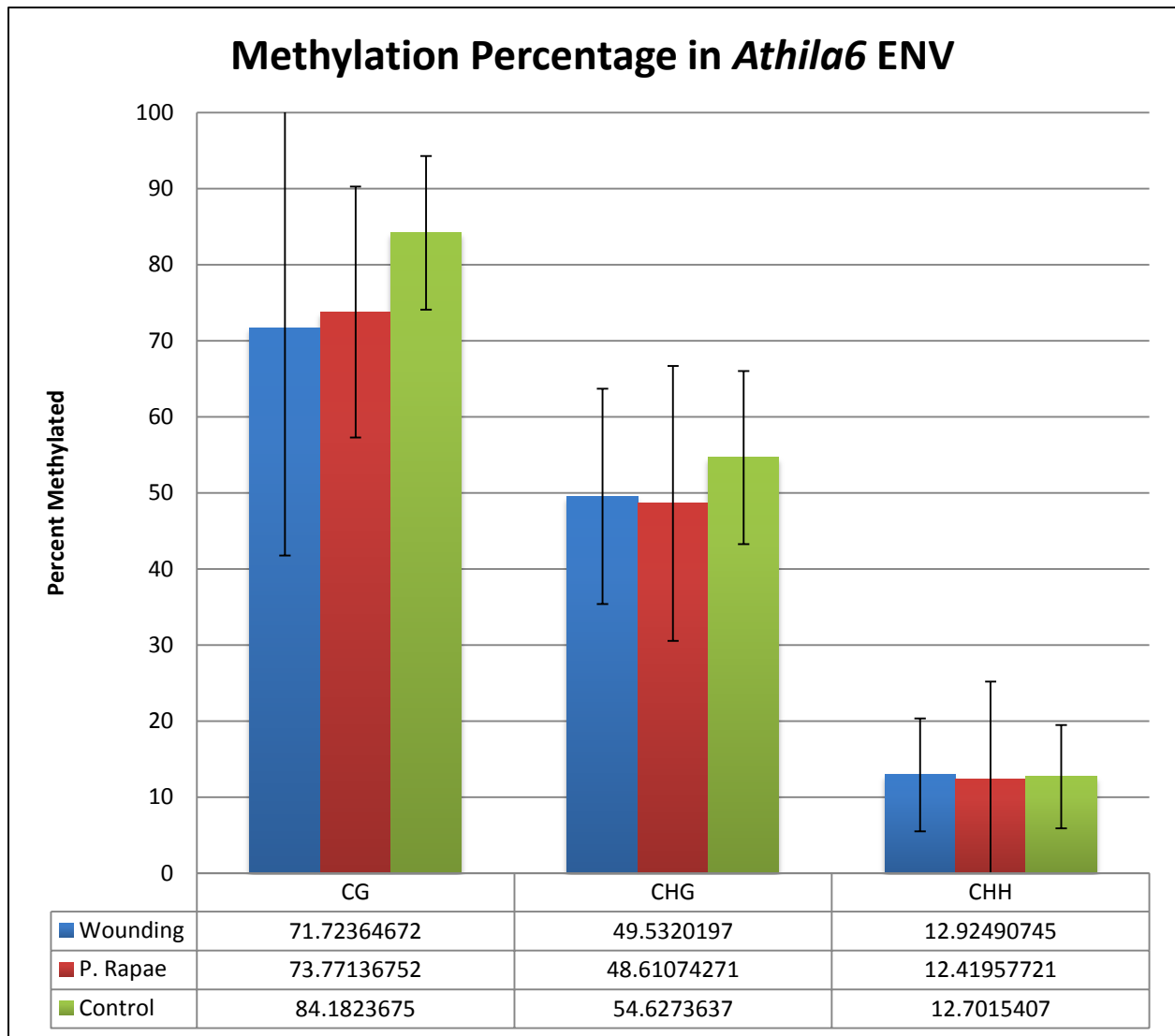


Fig 10. Bisulfite sequencing data concerning methylation of cytosine contexts in *Athila6* ENV region: The *Athila6* ENV region for each sample underwent bisulfite conversions and isolated for sequencing. The percentages of methylation for each cytosine context were averaged between samples allowing comparisons between the three different sample types.

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