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Epigenetic Regulation of Salicylic Acid-Mediated Plant Defense

By

Phillip Key

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors from the South Carolina Honors College

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Thesis Summary

The threat crop disease presents to modern agricultural systems has created an impetus for the study of how plants respond to pathogen challenge, and the investigation seeks ways that it might be changed and improved. For an immune response to be effective, an organism needs to recognize and react appropriately to the presence of a pathogen, and mutations can positively and negatively influence either of these processes. While many different proteins and their implications have been extensively documented in a common model organism like Arabidopsis, much of the influence that epigenetic variability has upon pathogen resistance remains largely unknown. The objective of this experimentation is to improve this condition by the identification of proteins related to epigenetic regulation that might have influence over the staging of an immune response in Arabidopsis thaliana plants. This identification can contribute to the developing catalog of studies that have attempted to resolve some of the effect that epigenetic variability has for pathogen resistance in Arabidopsis. Some such mutations might later be utilized in the creation of genetically modified plants that may have significantly altered immune responses.

To begin the project, specifically modified Arabidopsis plants were grown to the flowering stage from seed alongside Wild-Type and NPR1 knockout varieties. The first stage of experimentation with these plants involved an exploratory pathogen infiltration of ten different epigenetic mutants. In this process, hundreds of leaves were injected with a known concentration of Pseudomonas syringae pv. morsprunorum, and forty-eight hours later the amount of pathogen present in the leaves was quantified to evaluate the success of infection. From the results of the assays, a reduced number of mutant plants of interest were used for the
second stage of the project, which focused on analyzing the expression of a specific protein, the Nonexpressor of Pathogen Related Genes 1 (NPR1). A Western Blot was conducted on samples that had been exposed to the introduction of the plant hormone salicylic acid (SA), which is crucial for the development of NPR1 protein concentration. The presence of a high concentration of NPR1 protein is indicative of a highly effective pathogen response, and the Western Blot results were able to provide supporting evidence for the resistance to infection identified in the infiltration assays.

Mutant plants that showed an increased resistance to pathogen infiltration and a sufficient concentration of NPR1 protein after SA exposure were assessed for the expression levels of Pathogen Related Gene 1 mRNA. The quantification of PR1 was conducted by isolating the mRNA present in the plant samples, using reverse transcriptase to edit the mRNA transcripts present in the plant cells into cDNA, and preforming real-time PCR with specific primers to identify the abundance of cDNA encoding for PR1. A greater quantity of PR1 mRNA in a mutant relative to Wild-Type Col-0, similar to an increased concentration of NPR1 protein, is suggestive of a stronger immune response capability. When the infiltration results are considered, these data sets can provide a comprehensive picture of how individual epigenetic regulation-related mutations are affecting immune response.

From experimentation, two Arabidopsis mutants were identified as resistant to pathogen infiltration: sir2-1 and suvh456. In the pathogen infiltration stage, the quantity of *P. syringae* was significantly lower in these varieties than the Wild-Type plants, which prompted further investigation of the lines. The Western Blot for NPR1 results revealed that these mutants both had a high concentration of the protein after four hours of exposure to salicylic
acid, which supported the evidence from the infiltration assay. Finally, the $PR1$ mRNA transcript analysis revealed that the $sir2\cdot1$ and $suvh456$ were expressing much more $PR1$ relative to the control cDNA Ubiquitin 5 (UBQ5) than the Wild-Type, further reinforcing the idea that these plants were presenting a stronger pathogen response than the positive control. The identification of these mutants of interest allows for the conduction of additional research that seeks to characterize the specific effects these epigenetic regulation-related mutations have upon gene expression.
Abstract

Plant pathogens remain a significant threat to the stability of modern agricultural systems, and the investigation of mechanisms to improve the security of food resources has led to the partial characterization of plant immune response. With this progress, there is a newfound ability to analyze the relative pathogen resistance capability of specifically modified organisms, and in doing so, it is possible to identify individual alterations that might play a role in creating a more robust immune response. In this study, ten Arabidopsis thaliana mutants were infiltrated with Pseudomonas syringae pv. morsprunorum alongside positive and negative control Col-0 Wild-Type and npr1-2. The infiltrated mutant varieties that showed resistance to the development of infection by the pathogen were then analyzed for their the NPR1 protein content and concentration of PR1 mRNA. The results of both studies provided evidence that the suvh456 and sir2-1 mutants had a more effective immune response than the positive control Wild-Type variety, which supported the indication of pathogen resistance noted by the infiltration assay. These proteins have epigenetic influence over gene expression, and can be said to be acting as negative regulators of Arabidopsis immune defense.
Introduction

The Historical Impact of Crop Disease

Crop diseases can have enormous influence on the communities they impact, and some pathogen outbreaks have had noted historical significance. One such event was the Irish Potato Famine in the 1840s and early 1850s, which caused the death or emigration of over two million people, at least 24% of Ireland’s total population at the time. The famine was the result of the spread of the Potato Late Blight Disease (caused by the Phytophthora infestans), which shriveled plants and reduced the area covered by the potato crop from 2.1 million acres to .3 million acres in less than two years (Gráda 2000). Even to this day, the population of Ireland still has a significantly smaller population than before the famine (Phriomh-Oifig Staidrimh 2011). The outbreak was an imperative factor in the dispersal of Irish culture to the new world, the impact of which can still be experienced today in the St. Patrick’s Day festivities of major North...
America Cities (Moss 1995).

An Emerging Threat to Agricultural Production

Well over a hundred years later, despite significant advances in preparedness and understanding, the threat of crop disease continues to be a major challenge. Perhaps one of the most notable pathogens to impact food production recently is *Candidatus liberibacter*, which causes Citrus Greening Disease or Huanglongbing. Orange tree groves in Brazil and Florida have proven conducive to the spread of the disease, which causes mottled leaves and malformed fruit that falls from the tree too early (Gottwald et al. 2007, McClean 1970). Huanglongbing has become a significant threat to the agricultural production of oranges in Florida, an almost 9-billion-dollar industry. The harvest of oranges in Florida, because of Huanglongbing, is 23% lower than expected, and the condition has not shown significant improvement (Hodges et al. 2006). Even with the efforts of numerous researchers, problems like Huanglongbing can still

Figure 2. The mottled leaf of a Brazilian orange tree affected by Huanglongbing (Bové 2006).
have significant economic impacts and continue to disrupt the stability of critical food resources.

The Role of Salicylic Acid

Recently, advances in genetics and molecular biology have allowed for the general characterization of plant immune response. One critical part of this response that has been identified is the proper production of salicylic acid (SA), a hormone that signals cells to temporarily bolster their immune systems. Plants produce SA once a pathogen challenge has been identified, and the dissemination of this hormone causes leads to Systemic Acquired Resistance (SAR) in neighboring cells. Here, SAR refers to the increased expression of immune response-related proteins in response to interactions with SA, which reduces the resulting threat presented by the infiltration of a pathogen (Gaffney et al. 1993). SA causes the expression of Pathogen Related (PR) genes by binding to dozens of already identified SA Binding Proteins (SABP), and one of the most important of these when investigating immune response is the Nonexpressor of PR Genes 1 (NPR1) (Fu et al. 2012, Klessing et al. 2016). Because the immune response is so dependent of a numerous amount of proteins working appropriately, many genetic and epigenetic mutations can significantly alter the result of plant’s interaction with SA or influence another critical element of immune defense. This influence can either lead to an increased susceptibility or resistance to pathogen infiltration, and the immune systems of plants can be further characterized by studying the impact of specific mutations.

Plant Immune Response
In the presence of salicylic acid, the protein *NPR1* operates as a regulator of a plant’s SAR process by co-activating defense genes (Boatwright et al. 2013). Before this can happen, *NPR1* oligomers must first be reduced to a monomeric state and migrate to the nucleus from the cytosol, which occurs during the build up of SA. Without this reduction of *NPR1* proteins, defense gene expression does not take place, as the oligomers are not capable of entering the nucleus (Mou et al. 2003). WRKY binding proteins, which reside in the nucleus and are also activated by contact with SA, are utilized during an immune response in order to increase the concentration of *NPR1* protein. These proteins attach to the W box motif in the promoter region of the *npr1* gene to increase its expression and improve the effectiveness of an immune
defense (Yu et al. 2001). In the nucleus, NPR1 monomers cause transcription factors (TGA-bZIP) to form an enhanceosome, which positively regulates the expression of PR Genes 1, 2, & 5 (Després et al. 2000, Zhou et al. 2001, Fan et al. 2002). The breakdown of NPR1 in the nucleus occurs by proteasome-mediated degradation, and the expression of defense genes ceases without NPR1 monomers in the nucleus (Spoel et al. 2009).

**The Identification of Immune-Related Mutations**

By taking these molecular mechanisms into consideration, an experimental analysis of the relative effectiveness of the immune systems for different mutants of a plant species can be

![Figure 3](image.png) The influence of SA on NPR1 oligomers in the cytosol and WRKY binding proteins in the nucleus.
developed. This analysis involves the use of the common model pathogen *Pseudomonas syringae* as well as the plant model *Arabidopsis*, and together these can demonstrate the influence that specific genetic mutations in *Arabidopsis* might have on immune response. The most theoretically straightforward attempt to quantify this effectiveness is perhaps a pathogen infiltration assay, where a known concentration of *P. syringae* is injected into the leaves of *Arabidopsis* mutants and the abundance of pathogen in the leaves is assessed a few days later. Plants with lower pathogen counts are candidates for further testing, which involves assessing the level of *NPR1* protein present after exposure to SA. By spraying the SA hormone onto the plants, the SAR response can be mimicked, and plants begin produce additional *NPR1* because of SA’s interaction with the WRKY binding proteins. A high concentration of *NPR1* protein relative to a control Wild-Type, which can be determined by extracting the protein composition of leaf samples and conducting a Western Blot with *NPR1* antibodies, is a secondary indication of an effective immune system. The presence of reduced NPR1 proteins in the cell promotes the transcription of *PR1* genes, which provides a third verification of the relative magnitude of immune response for the *Arabidopsis* mutants. The abundance of these *PR1* mRNA transcripts can be assessed by isolating the mRNA, turning that mRNA into cDNA with reverse transcriptase, and preforming real-time PCR on the resulting cDNA. Mutant *Arabidopsis* plants that demonstrate a decreased quantity of pathogen in the infiltration assay, an increased concentration of *NPR1* protein in the Western Blot, and an increased concentration of *PR1* gene transcripts from the rtPCR compared to the positive control Wild-Type varieties can be said to contain an enhanced immune response.
Materials and Methods

Growing Arabidopsis thaliana

During this project, the following three processes were used in data collection: Pathogen Infiltration, Protein Western Blot, and rtPCR Gene Analysis. In order to create the samples necessary for the experimentation, seeds from positive and negative control Arabidopsis varieties (Col-0 WT, and NPR1 knockout npr1-2) were sown alongside ten homologous mutants. After planting the Arabidopsis seeds, the growing trays were placed into a 4°C Celsius room for two days to promote germination. These trays were then moved to an appropriate space in a growing chamber, which received twelve hours of fluorescent light a day. When the mutants had reached a sufficient transplantation size of approximately one centimeter after two weeks, ten members of each mutant were moved and spread out in a new set of trays. The newly transplanted seedlings were watered twice a week, and after three more weeks, began to approach the flowering stage.

Before the plant varieties began to flower, two 0.1g leaf samples were collected from each, and these were then frozen in liquid nitrogen. Next, the plants were evenly misted with a solution of 0.5mM salicylic acid and covered to increase humidity. The collection process was repeated after the plants had been exposed to SA for four and twenty-four hours, and the resulting samples were taken from the liquid nitrogen to be frozen at -80°C.
Pathogen Infiltration

Infiltrant Preparation

Two pathogen infiltrations were conducted during this study, containing five and then eight plants to improve error bars. To begin the process, plants nearing flowering (approximately five weeks old) were watered and covered for six hours in order to increase the permeability of the leaves. During this time, 100mg/L of the antibiotic streptomycin was added to a solution of King’s B medium [Protease Peptone #2 (DIFCO), K2HPO4, glycerol, MgSO4]. Next, part of a frozen Pseudomonas syringae pv. morsprunorum colony (PSM ES 4326) was added to the solution as well, and it was incubated for twenty-four hours at 30°C. When ready, this solution was centrifuged to separate the P. syringae from solution, and an aspirated sample of the P. Syringae was suspended in 10Mm MgCl2 at an absorbance value of 0.001 OD600nm. Once the infiltrant had been created and standardized, a blunt-tip syringe (no needle) was used to inject it into two similar marked leaves of each plant. After infiltration, the trays were brought back into the grow chambers, and the air flow was stopped to increase the humidity. While the infiltration took place, an appropriate number of plates were created by adding bacteriological

Figure 4. Pathogen infiltrated leaves during the collection stage, specifically from the Col-0 Wild-Type.
agar to additional King’s B medium, and these were kept sterile.

*Pathogen Abundance Quantification*

Forty-eight hours later, the infiltrated leaves were cut from the plants, photographed, and submerged in individual screw-top tubes containing 10Mm MgCl₂ as well as a small sterile steel ball. These samples were taken to the GenoGrinder®, where there were processed at 1200 strikes/min for three minutes. The resulting solution contained suspended *P. Syringae* and it was diluted with 10Mm MgCl₂ at six decreasing magnitudes of concentration from 10⁻¹ to 10⁻⁶. For every mutant organism sampled, 10µL of each dilution was placed on a King’s B agar plate using a multi-channel pipette, and the solution was allowed to run down the plate. The resulting plates were incubated for two days at 30°C, and the number of colonies for each dilution were counted afterwards.

*Figure 6.* The proliferation of *P. syringae* colonies at different magnitudes of dilution on a King’s B agar plate.
NPR1 Western Blot

Protein Extraction

For the Western Blot process, the frozen samples collected four hours after exposure to SA were crushed in the GenoGrinder® at 1000 strikes/min for two minutes. 100µM of a protein extraction buffer solution was added to the samples for each 0.1g of processed leaf, and the samples were vortexed for ten seconds and placed on ice. The samples were centrifuged twice at 4°C and 13,500g for 15 minutes each time. The supernatants of the centrifuged samples were removed and placed into separate tubes, and Laemmli Sample Buffer 1/5 the volume of the removed supernatants was added to the tubes. These tubes were then placed into a 75°C water bath and heated for ten minutes.

<table>
<thead>
<tr>
<th>Protein Extraction Buffer Components</th>
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<tbody>
<tr>
<td>Protease Inhibitor Cocktail</td>
</tr>
<tr>
<td>Proteasome inhibitor MG115</td>
</tr>
<tr>
<td>DTT</td>
</tr>
<tr>
<td>PMSF</td>
</tr>
<tr>
<td>Tris-HCl</td>
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<tr>
<td>NaCl</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Triton X-100</td>
</tr>
<tr>
<td>IGEPAL</td>
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<tr>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

To begin the Western Blot Process, an electrophoresis apparatus was constructed by pouring 1x MOPS Running Buffer into a buffer tank. A 4-12% polyacrylamide gel was loaded into the tank, and 150V was applied to the tank for sixty minutes. After the protein had migrated for the appropriate length of time, the gel was carefully removed from its casing and submerged
into Tris-Bicine Transfer Buffer where it was placed onto the positive side of a submerged gel holder cassette with foam pads and Watman Paper. A nitrocellulose membrane was added on the positive side of the cassette, and the cassette was closed before being placed into a chilled (transfer) buffer tank. An electrical current of 100V was applied to the tank for 90 minutes, and the cassette was removed from the apparatus. During the transfer, a blocking solution had been created with non-fat milk concentrate (5%), 1x Tris-Buffered Saline (TBS), and Tween (0.1%). The membrane, which now contained the transferred protein bands from the gel, was carefully separated from the cassette and washed in TBS for 10 minutes.

After washing, the membrane was placed into 5mL of the blocking solution and placed onto the shaker for an hour. The membrane was washed again three times for five minutes in TBS-T (TBS with Tween) when the blocking step had completed. A primary antibody, anti-NPR from Agrisera, was added at a 1 to 1000 dilution to the blocking solution, and the membrane was submerged and placed onto the shaker overnight. In the morning, after another washing phase, the membrane was placed into a blocking solution that contained the antibody conjugate, goat anti-rabbit, at a 1 to 5000 dilution. After a final five-minute wash, the membrane was submerged in a 1: dilution of West Dura Extended Duration Substrate for the development of chemiluminescence. X-ray film was exposed to the membrane in a dark room, and the developed film demonstrated the presence of NPR1 in the membrane. Ponceau S dye was then added to the membrane, which was recorded along with the x-ray film.
**PRI Gene Analysis**

**Genetic Isolation**

In order to analyze the influence of SA on the expression of PRI1 mRNA in the mutants, frozen samples from before SA and twenty-four hours after SA collection utilized. The samples were placed into the GenoGrinder® at 1200 strikes/min for three minutes, and 1mL of Trizol was added to the processed samples. The tubes were vortexed extensively, left at room temperature for five minutes, and finally centrifuged at 4°C and 12000rpm for ten minutes. The supernatant was removed from the tubes with the leaf debris, and added to additional tubes that contained 200µL of chloroform. The samples were once again vortexed, left at room temperature for three minutes, and spun in the centrifuge at the same settings as before. The sample supernatants from this second centrifuging were removed and placed into a third set of tubes that contained 600µL of isopropanol. After inverting the tubes and leaving them at room temperature for ten minutes, the tubes were centrifuged one final time.

**mRNA Isolation, Reverse Transcriptase, and rtPCR**

At this point, a solid DNA and RNA pellet was isolated at the bottom of the spun tubes, and the liquid portion was removed. 1mL of 75% EtOH was added to the tubes, and they were centrifuged once again at 4°C and 7500 rpm for five minutes. The EtOH solution was removed, and the tubes were inverted in an incubator at 37°C to dry for thirty minutes. 30µL of DEPC-H₂O was used to re dissolve the dried pellets, ad they were placed into a 55°C water bath for 10 minutes. The RNA concentration of the solution was determined using OD₂₈₀nm specially configured to correct for the presence of DNA in the sample.
A volume of sample that was equivalent to 1µg of RNA was added to a solution containing 1x reaction buffer with MgCl\(_2\), 1x of DNase1, and DEPC water was added until each samples volume was 10µL. The samples were incubated at 37°C for a half hour, and 1µL of 50mM EDTA was included before a second incubation at 65°C for ten minutes. At this point, the RNA samples were ready for reverse transcription, and could be added to .2L micro-tubes that contained 4µL 5x qScript cDNA Supermix, and DEPC-H\(_2\)O for a total volume of 20µL. The micro-tubes were centrifuged and incubated for 5 minutes at 25°C, 30 minutes at 42°C, and then finally five minutes at 85°C. The samples were diluted and placed in a 96-well plate with the forward and reverse primers for UBQ5 or NPR1 as well as 1x Syber Green Supermix, and the 96-well plate was placed into the rtPCR machine for analysis.
Results
Pathogen Infiltration

Figure 7. The upper two charts depict *Pseudomonas syringae pv. morsprunorum* abundance by *Arabidopsis* mutant as quantified by the first (left) and second (right) infiltration assays, along with the 95% confidence intervals for the true mean values. In the lower left figure, the typically observed pathogen symptoms for the positive and negative controls (Col-0 and *npr1-2*) are compared to those of the *suvh456* and *sir2-1* mutants. The quantification of pathogen abundance for these four varieties from the second trial is showcased on the lower right.
**Figure 8.** The above portion of this figure shows the protein concentration of the unspecific band as revealed by a Ponceau S Stain. In the Western Blot Results (below) a marker has been added to the location where the NPR1 70kDa marker from the protein ladder had migrated to on the gel. The positive and negative controls for the expression of NPR1 (Col-0 and npr1-2) are displayed next to the two Arabidopsis mutants of interest (suvh456 and sir2-1) in the figure.
rtPCR Concentration Analysis of PR1

<table>
<thead>
<tr>
<th>Mutant</th>
<th>0 SA PR1/UBQ5</th>
<th>24 HR SA PR1/UBQ5</th>
</tr>
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<tbody>
<tr>
<td>Col-0</td>
<td>0.00883023</td>
<td>2.084931522</td>
</tr>
<tr>
<td>npr1-2</td>
<td>0.000169081</td>
<td>0.020880981</td>
</tr>
<tr>
<td>suvh456</td>
<td>0.000101704</td>
<td>12.26662676</td>
</tr>
<tr>
<td>sir2-1</td>
<td>0.006912069</td>
<td>18.18907157</td>
</tr>
</tbody>
</table>

Table 2. In this table, the mean values for the ratio of PR1 cDNA to the control UBQ5 cDNA from rtPCR are documented for the Arabidopsis varieties before and 24 hours after SA exposure.

Figure 9. A graphical interpretation of the differences in PR1/UBQ5 cDNA produced. Here, the error bars represent the total range of values produced by rtPCR for the PR1 cDNA content of the samples.
Discussion

The results of this project present a cohesive depiction of the influence that two epigenetic regulation-related mutations of interest, \textit{sir2-1} and \textit{suvh456}, have upon the immune response of \textit{Arabidopsis thaliana}. These mutants were chosen for further study because of the results of the first stage of experimentation, where an exploratory pathogen infiltration assay was conducted with \textit{Pseudomonas syringae pv. morsprunorum}, which suggested that after 48 hours they contained merely a fraction of the pathogen that was present in the positive control Col-0. This result held up to a second round testing, where the results were approximately the same relative to Col-0. Additionally, these mutants consistently had a reduction in the observable symptoms of pathogen infection compared to Col-0 as well. The \textit{sir2-1} and \textit{suvh456} mutants were seemingly able to mount an immune defense that was superior to the Wild-Type variety, and further testing began to validate these findings.

The second stage of experimentation involved the isolation and analysis of the \textit{NPR1} protein composition after a 4-hour exposure to SA using a Western Blot. Here, the protein content of the positive control Col-0 and the negative control \textit{npr1-2} was compared to that of the mutants. As expected, the Col-0 Wild-Type samples results suggested the presence of \textit{NPR1} protein, and the \textit{npr1-2} mutants showed a distinct lack of \textit{NPR1} presence at the marker denoted by the \textit{NPR1} protein ladder. The \textit{sir2-1} mutant had the darkest band, which was suggestive of the greatest relative \textit{NPR1} protein concentration. The \textit{suvh456} mutant had approximately the same pigmentation as the Col-0 Wild-Type samples, but was a much broader band, which might suggest a greater protein content. This discrepancy suggests the need for further testing. Additionally, the total protein loaded into the gels during the experiment was assessed for each
variation using a Ponceau S stain, which demonstrated that a similar amount of protein was added to each sample.

For the third stage and final stage of experiments, reverse transcriptase and real-time PCR was utilized to detect the presence of PRI gene sequences in the cDNA derived from the isolated mRNA. Before treatment with SA, every variation of Arabidopsis was shown by rtPCR to have had a very small ratio of PRI cDNA to the control UBQ5 cDNA. After treatment, however, the Col-0, sir2-1, and suvh456 varieties had a large increase in the presence of PRI cDNA relative to the UBQ5. The sir2-1 and suvh456 mutants both had a PRI cDNA expression level that was almost a magnitude larger (relative to the UBQ5 expression) than the positive control Wild-Type variety, which was in line with the assumption that these mutants had more effective immune responses. The suvh456 plants had the greatest ratio of PRI to UBQ5, which was consistent for every sample tested. This increased expression of PRI mRNA in the two mutants of interest provided additional evidence for their increased immune response capability.

Because the sir2-1 and suvh456 mutants proved to improve the immune response of Arabidopsis plants, an effort was made to build an understanding of their influence on gene expression. The sir2-1 mutant is a T-DNA knockout mutant of the silent information regulator protein (SIR2 or AtSRT2), which involved in the deacetylation of histones. The deacetylation of histones causes the tightening of chromatin complexes, and causes the repression of genes (Cress et al. 2000). Because of its ability to modify histones, SIR is capable of silencing the transcription of DNA in chromatin, and repressing the expression of genes related to processes related to the cell cycle such as aging and apoptosis (Wang et al. 2010). The suvh456 mutant is a triple mutant that prevents the function of three SUVH proteins, which act as histone methyltransferases. In the modified mutants, there is an absence of methylation at the H3K9
locus, as well as non-CG methylation (Ebbs et al. 2006). In Arabidopsis plants, the methylated H3K9 locus is used for the formation of heterochromatin complexes, which prevent the transcription of DNA (Jackson et al. 20002). The results of this study support the conclusion that by condensing the chromatin of defense genes, the SIR2 and SUVH proteins are acting as negative regulators for plant immune defense.
References


