The role of RNA silencing in plant defense against the fungal pathogen Fusarium oxysporum in Arabidopsis thaliana

Sameer Tiwari
University of Wollongong

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The role of RNA silencing in plant defense against the fungal pathogen *Fusarium oxysporum* in *Arabidopsis thaliana*

Sameer Tiwari

A thesis submitted for the degree of Doctor of Philosophy of the University of Wollongong, June, 2013
DECLARATION

Except where specific reference is made to other sources, the work presented in this thesis is the work of the author. It has not been submitted, in whole or in part for any other degree.

Sameer Tiwari
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DEDICATION

To my parents, wife and late grandparents for their support and encouragement.
ABSTRACT

Plants defend their genomes by using RNA–directed DNA methylation (RdDM), an epigenetic mechanism driven by small interfering RNAs (siRNAs) to repress parasitic invaders. In Arabidopsis thaliana, DNA glycosylases of the DEMETER (DME) family prune cytosine methylation from DNA. Demethylation by DME in Arabidopsis is necessary for gene imprinting while DNA demethylation by the DEMETER-Like (DML) enzymes and REPRESOR OF SILENCING1 (ROS1) removes silencing directed by RNA silencing pathways. Prior to this work, the role of demethylases in plant defense was not known. Moreover, the role of the RdDM pathway in plant defense against non viral pathogens is poorly understood.

Here in a genetic screen designed to characterize RNA silencing factors regulating plant defense in Arabidopsis thaliana, against the root infecting fungal pathogen Fusarium oxysporum, we identified downstream factors of the RdDM pathway, RNA Polymerases V (PolV) and ARGONAUTE 4 (AGO4) that are critical for plant defense. In addition, the novel role of DNA demethylases in plant defense was also revealed.

The mutant plants deficient in DNA methylation and demethylation factors showed increases in disease susceptibility to F. oxysporum. By employing microarray techniques, we found mis-expression of a large number of genes in mutant plants deficient in methylation and demethylation factors relative to the wild-type plants. A large proportion of these genes, especially in the demethylase mutant, are associated with biotic stress. These results suggest that RdDM and DNA demethylation play a critical role in plant disease resistance. Furthermore, my
results show that the RdDM pathway factor PolV negatively regulates the expression of *PATHOGENESIS RELATED 1 (PRI)* gene, a molecular marker of the salicylic acid defense pathway (SA), suggesting the involvement of epigenetic mechanisms in modulating defense signaling pathways.

Taken together my results highlight the importance of two antagonistic mechanisms: DNA methylation and demethylation in the regulation of plant immunity against the fungal pathogen *F. oxysporum* and perhaps against other biotic and abiotic stresses.
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ABBREVIATIONS

Agrobacterium  Agrobacterium tumafacines
Arabidopsis  Arabidopsis thaliana
AGO  Argonaute-like protein
Botrytis  Botrytis cinerea
CMT3  CHROMOMETHYLASE 3
Col-0  Columbia-0 (Arabidopsis thaliana ecotype)
DCL  DICER-LIKE
DML  DEMETER LIKE
DRB  Double-stranded RNA binding protein
DRD  DEFECTIVE IN RNA-DIRECTED DNA METHYLATION
DRM  DOMAINS REARRANGED METHYLASE
dsRNA  double stranded RNA
DTT  Dithiothreitol
Dpi  days post infection
EtBr  Ethidium bromide
Fusarium  Fusarium oxysporum
HDA  Histone deacetylase
HEN1  HUA ENHANCER 1
HYL1  HYPNOSTIC LEAVES 1
IPTG  Isopropyl β-D-1-thiogalactopyranoside
JA  Jasmonic acid
Kan  Kanamycin
LB  Luria-Bertani broth
Ler  Landsberg erecta (Arabidopsis thaliana ecotype)
MET1  Methyltransferase 1
MS  Murashige and Skoog plant growth media
nat-siRNA  Natural antisense small interfering RNAs
PAZ  Piwi–Argonaute–Zwille
PDB  Potato dextrose broth
PDF  PLANT DEFENSIN
Pol V  DNA-dependent RNA polymerase V
PPR  Pentatricopeptide repeat
PRI  PATHOGENESIS RELATED 1
*Pseudomonas*  *Pseudomonas syringae*
qRT-PCR  Quantitative RT-PCR
ra-siRNA  Repeat associated small interfering RNA
RdDM  RNA-directed DNA methylation
RDR  RNA-dependent RNA polymerase
Rif  Rifampicin
RISC  RNA-induced silencing complex
RNAi  RNA interference
ROS1  REPRESSOR OF SILENCING 1
RT-PCR  Reverse transcription PCR
SA  Salicylic acid
SDS  Sodium dodecyl sulphate
SDG  SET DOMAIN GROUP
SE  SERRATE
SGS3  SUPPRESSOR OF GENE SILENCING 3
siRNA  small interfering RNA
TAS  ta-siRNA gene
TAIR  Arabidopsis Information Resource database
TF  Transcription factors
ta-siRNA  trans-acting siRNAs
T-DNA  Transfer DNA (in the tumour-inducing plasmid of *Agrobacterium*)
VSP  VEGETATIVE STORAGE PROTEIN
CHAPTER 1: Literature Review
1.1 Introduction

The phenomenon of RNA silencing was first observed in 1990, when transformation of petunia with a transgene designed to increase expression of a pigment biosynthesis enzyme unexpectedly lead to a loss of flower pigmentation, where expression of both the transgene and endogenous gene were suppressed (Napoli et al., 1990; van der Krol et al., 1990). Initially termed “cosuppression”, the phenomenon was shown to be mediated by sequence-specific RNA degradation and associated with de novo DNA methylation (Jones et al., 1998). In the mid 1990’s, research on pathogen-derived resistance to viruses in plants demonstrated that viral infection could trigger sequence-specific RNA degradation, which in turn inhibited viral replication, providing early evidence of RNA silencing as a plant antiviral defense mechanism (Lindbo et al., 1993). RNA silencing has become a major focus of molecular biology and biomedical research since 1998, when dsRNA was demonstrated to be an effective trigger of silencing in both animals and plants (Fire et al., 1998; Waterhouse et al., 1998). Over the past ten years, significant progress has been made in the characterization of the molecular mechanisms and biological roles of RNA silencing in both plants and animals (Baulcombe, 2004; Ambros, 2004). Significant mechanistic insight has been gained using model experimental systems such as injection of dsRNA into C. elegans, in vitro RNA assays of Drosophila embryo extracts, and molecular and phenotypic analysis of Arabidopsis insertional mutants, and the spectrum of biological processes in which RNA silencing has been shown to play a role is rapidly expanding.

In addition to antiviral defense, RNA silencing plays a key role in the control of development and maintenance of genome stability in both plants and animals. Recent evidence also indicates
that RNA silencing plays an important role in stress responses and disease development (Dunoyer et al., 2006; Katiyar-Agarwal et al., 2006).

This literature review aims to (i) provide a brief overview of RNA silencing pathways in plants, (ii) discuss the biological functions RNA silencing in plants, including antiviral defense; and (iii) summarize the advances and recent evidence for the involvement of RNA silencing in plant defense against non-viral pathogens.

1.2 Small RNA pathways in plants

In plants, RNA silencing is triggered by double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) of different sizes and origins (Meister and Tuschl, 2004). The trigger dsRNA molecules can be synthesized by RNA-templated RNA polymerization or hybridization of complementary transcripts. The dsRNAs or hpRNAs are processed by DICER-LIKE (DCL) proteins into 21-25 nt small RNAs, which form RNA silencing effector complexes with members of the ARGONAUTE (AGO) protein family, to guide cleavage or translational inhibition of complementary single-stranded RNAs or direct cytosine methylation of homologous DNA, as shown in Figure 1.1 (Baulcombe, 2004; Meister and Tuschl, 2004). Small RNAs can be classified as exogenous small interfering RNAs (siRNAs), repeat-associated siRNAs (ra-siRNAs), trans-acting siRNAs (ta-siRNAs), natural cis-antisense siRNAs (nat-siRNAs), long siRNAs (lsiRNAs) and microRNAs (miRNAs) on the basis of their origin and function (Table 1).
Four DCL proteins have been identified in *Arabidopsis*. DCL2, DCL3 and DCL4 process dsRNA into various types of siRNA; DCL2 produces 22 nt viral and nat-siRNAs, DCL3 produces 24 nt ra-siRNAs, and DCL4 produces 21 nt viral siRNAs, ta-siRNAs and some miRNAs (Xie et al., 2004; Gasciolli et al., 2005). DCL1 functions in conjunction with the dsRNA-binding domain (dsRBD) protein HYL1 (or DRB1) and another factor, SERRATE, to process miRNA precursors (Vazquez et al., 2004; Lobbes et al., 2006). Biogenesis of small RNAs in plants also requires the common factor HUA ENHANCER 1 (HEN1) (Boutet et al., 2003), which adds a methyl group to the 2-O-hydroxyl group of the 3’ terminal nucleotide of mature siRNAs and miRNAs, to protect small RNAs from degradation and polyuridylation (Papp et al., 2003; Li et al., 2005; Yu et al., 2005). Unlike mammalian miRNAs, which are processed through a series of nuclear-cytoplasmic steps involving two different RNaseIII-like enzymes, plant miRNAs appear to be only processed in the nucleus by a single DICER, DCL1 (Han et al., 2004; Park et al., 2005). It is unclear how different DCLs recognize different dsRNAs; however, the different subcellular localization and varying interactions of DCLs with specific dsRBD proteins may partly regulate the recognition of dsRNA. Indeed, five different dsRBD proteins have been identified in *Arabidopsis* (Hiraguri et al., 2005), including HYL1 and DRB4 which interact with DCL1 and DCL4, respectively. DCL1 and DCL3 function in the nucleus, while DCL2 and DCL4 appear to act in both the nucleus and cytoplasm (Finnegan et al., 2003; Hiraguri et al., 2005).
Figure 1: Schematic representation of the parallel DCL/sRNA-directed RNA silencing pathways in the model dicotyledonous species *Arabidopsis*. 
Table 1.1: Classification and function of endogenous small RNAs and the proteins involved in small RNA biogenesis in plants.

<table>
<thead>
<tr>
<th>Small RNA</th>
<th>Size (nt)</th>
<th>DCL1</th>
<th>DCL2</th>
<th>DCL3</th>
<th>DCL4</th>
<th>RDR2</th>
<th>RDR6</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>miRNA</td>
<td>21-24</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Endogenous gene expression</td>
</tr>
<tr>
<td>ta-siRNA</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Phase transition, mobile developmental signaling</td>
</tr>
<tr>
<td>la-siRNA</td>
<td>30-40</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Biotic stress responses</td>
</tr>
<tr>
<td>nat-siRNA</td>
<td>21-24</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Biotic and abiotic stress responses</td>
</tr>
<tr>
<td>ra-siRNA</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>De novo DNA methylation and chromatin silencing</td>
</tr>
</tbody>
</table>

The size corresponds to the major accumulating species of small RNA. “+” indicates a requirement for the enzyme in the respective small RNA biogenesis pathway.
1.3 siRNA-mediated silencing of viruses and transgenes

The siRNA pathway responsible for silencing viruses and transgenes in plants involves the RNA-dependent RNA polymerases RDR6 and/or RDR1 (Dalmay et al., 2000; Mourrain et al., 2000; Xie et al., 2001; Diaz-Pendon et al., 2007). However, it has also been suggested that some viral siRNAs are derived from direct processing of stem-loop structures formed within single-stranded viral RNAs by Dicer (Molnar et al., 2005), which may occur via a mechanism independent of RDR activity. Similarly, hairpin RNAs (hpRNA) expressed from inverted-repeat (i/r) transgenes are also a direct substrate of Dicer (Dunoyer et al., 2005; Fusaro et al., 2006). RDRs convert single-stranded RNAs into dsRNAs, with or without siRNA as a primer, which triggers or amplifies RNA silencing in plants (Tang et al., 2003; Moissiard et al., 2007; Voinnet, 2008). The features of RNA which serve as a template for RDRs are not yet fully characterized; however, cleavage products of the RNA-induced silencing complex (RISC) may be a candidate. For example, the ta-siRNA transcript is initially cleaved by miRNAs, and then serves as template for RDR6 to synthesize dsRNA, resulting in ta-siRNAs (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). RDRs also exist in fission yeast, fungi and C. elegans (Cogoni and Macino, 1999; Sijen et al., 2001), however, RDRs have not yet been detected in insects or mammals, although a non canonical RdRP has been recently identified in Drosophila (Lipardi et al., 2009).

Viral dsRNA processing is mainly catalyzed by DCL4, and to a lesser degree by DCL2 (Deleris et al., 2006; Fusaro et al., 2006). However, biogenesis of siRNAs from DNA viruses also involves DCL3 as indicated by co-localization of viral RNA intermediates and DCL3 in the nucleus. The hpRNAs derived from inverted-repeat (i/r) transgenes resemble viral RNAs, and are
mainly processed by DCL4 and DCL2 (Deleris et al., 2006; Fusaro et al., 2006; Moissiard and Voinnet, 2006). In some instances, expression of i/r transgenes is also associated with accumulation of 24 nt siRNAs, indicating the involvement of DCL3 in hpRNA processing (Fusaro et al., 2006).

The systemic nature of siRNA-mediated transgene silencing in plants is an interesting phenomenon. Transgene silencing can spread from cell to cell and over long distances through the plant vascular system (Voinnet, 2005). Systemic silencing requires DCL4 and RDR6, suggesting the involvement of 21 nt siRNAs in the signaling process, and a requirement for RDR6 in amplification of the signals (Dunoyer et al., 2005; Voinnet, 2005). Surprisingly, some of the factors involved in RNA-directed DNA methylation (RdDM) are also required for graft-transmission of transgene silencing, indicating that chromatin modification plays a role in the perception and perpetuation of long-distance silencing signals (Brosnan et al., 2007). The movement of small RNAs may play a role in antiviral defense in plants, as viral siRNAs may spread both locally and systemically ahead of the virus to provide early viral defense.

1.4 Natural antisense siRNAs as specific regulators of stress response in plants

Nat-siRNAs are a class of endogenous siRNAs which was recently discovered in Arabidopsis, and are derived from pairs of natural cis-antisense transcripts (Borsani et al., 2005). One transcript of the first identified nat-siRNA (P5CDH) is constitutively expressed, whereas the other transcript (SRO5) is induced by salt stress. When both transcripts are present a stable 24 nt siRNA, derived from the region of complementarity, is produced through the action of DCL2, NRPD1a (PolIVa), RDR6 and SGS3 (Borsani et al., 2005). The 24 nt siRNA guides cleavage of
the constitutive transcript, inducing production of 21 nt nat-siRNAs by DCL1 and further cleavage of the constitutive transcript (Borsani et al., 2005). Another recently identified nat-siRNA, nat-siRNAATGB2, is induced by the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis* (Borsani et al., 2005). Biogenesis of siRNAATGB2 requires DCL1, HYL1, HEN1, RDR6, NRPD1A and SGS3. The 22-nt siRNA, nat-siRNAATGB2 downregulates the pentatricopeptide repeat-like gene (*PPRL*) gene which contains complementary binding sites and is a negative regulator of the RPS2 disease resistance pathway, leading to enhanced resistance against *P. syringae* in *Arabidopsis* (Katiyar-Agarwal et al., 2006). The detection of nat-siRNAs under both abiotic and biotic stress conditions indicates that nat-siRNAs play a general role in the stress response of plants.

1.5 MicroRNAs and ta-siRNAs as endogenous regulators of gene expression

siRNAs are *cis*-acting, as they are derived from viruses, transgenes and transposons, and degrade source RNA molecules. In contrast, miRNAs and ta-siRNAs do not originate from the genes they regulate (Bartel, 2004), and miRNAs are derived from endogenous transcripts which contain short complementary inverted repeats and from hairpin-like dsRNA (Bartel, 2004). The principal mode of action of plant miRNAs is similar to siRNAs, primarily targeting the coding region and reducing gene expression via mRNA cleavage (Jones-Rhoades et al., 2006). In contrast, animal miRNAs primarily bind to the 3’ untranslated regions (UTRs) of target genes to suppress gene expression via translational inhibition (Ambros, 2004). However, a recent study suggested that translational repression may occur more commonly during plant miRNA-mediated regulation than previously thought (Brodersen et al., 2008).
Most plant miRNAs regulate transcription factors which are required at various stages of plant development (Jones-Rhoades et al., 2006). The importance of miRNAs during development was first realized when developmental abnormalities were observed in miRNA pathway mutants, such as the carpel factory (caf), short integuments 1 (sin1), suspensor (sus1) and embryo defective 76 (emb76) mutants which contain mutations in the DCL1 gene (Golden et al., 2002). Likewise, the hyponastic leaves 1 (hyl1) mutant displays impaired hormone response and flower development, and the transition from the juvenile to adult vegetative phase is delayed in the hua enhancer 1 (hen1) mutant (Lu and Fedoroff, 2000). In addition to transcription factors, plant miRNAs have also been shown to target ATP-sulfurylases, superoxide dismutases, laccases and ubiquitin conjugating enzymes (Bartel, 2004; Jones-Rhoades and Bartel, 2004).

Ta-siRNAs are derived from the long primary transcripts which are targeted by miRNAs. The biogenesis of ta-siRNAs therefore requires AGO1, DCL1, HEN1 and HYL1. Additionally, SGS3 and RDR6 are also essential for converting one of the two miRNA-cleaved TAS RNA fragments into dsRNA (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). The dsRNA is subsequently processed by DCL4 in a phased manner to generate clusters of ta-siRNAs (Vazquez et al., 2004; Allen et al., 2005; Yoshikawa et al., 2005).

In a similar manner to miRNAs, ta-siRNAs can regulate the expression of endogenous genes via RNA cleavage (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). Different members of the same gene family can be targeted by both miRNAs or ta-siRNAs. For example miR161 and the TAS2 ta-siRNAs target pentatricopeptide repeat protein (PPR) family members (Rhoades et al., 2002; Allen et al., 2005; Yoshikawa et al., 2005). Similarly, miR160,
miR167 and TAS3 ta-siRNAs target members of the auxin response factor (ARF) family (Rhoades et al., 2002; Allen et al., 2005). Some ta-siRNAs can act in the nucleus, as a number of TAS1a-derived ta-siRNAs have sequence homology with the intron of the At2g46740 pre-mRNA, which encodes a flavin adenine dinucleotide (FAD)-binding domain, and ta-siRNA-deficient mutants express high levels of unspliced At2g46740 RNA in the nucleus (Vazquez et al., 2004). Ta-siRNAs have also been shown to be involved in mobile developmental signaling (Ji et al., 2011). AGO1 may be responsible for slicing mRNAs targeted by TAS1 ta-siRNAs, as TAS1 ta-siRNAs associate with AGO1 in vitro (Baumberger and Baulcombe, 2005) and AGO1 and AGO7 have been implicated in TAS2- and TAS3-mediated regulation, respectively (Adenot et al., 2006).

1.5.1 RNA-directed DNA methylation

Repetitive DNA sequences, such as transposable elements in the plant genome, are frequently associated with 24 nt siRNAs (ra-siRNAs) which direct cytosine methylation and chromatin modification (Zhang et al., 2006). RNA-directed DNA methylation (RdDM) was first observed with transgenes in plants in 1994 (Wassenegger et al., 1994), and siRNAs generated by viruses and transgenes can also direct RdDM in plants (Mette et al., 2000; Wang et al., 2001). These siRNAs, known as repeat-associated or heterochromatic siRNAs, direct RdDM by providing the sequence specificity necessary for multiprotein complexes to bind and methylate their target DNA sequences. A number of different protein factors are required for upstream biogenesis of the 24-nt siRNAs and downstream de novo cytosine methylation during RdDM (Matzke et al., 2009). The proteins involved in RdDM include two plant-specific DNA-dependent RNA polymerases, PolIV and PolV. PolIV and PolV specifically function during different steps of the
RdDM pathway and are not essential for plant viability, as the loss-of-function mutants display normal growth under controlled environments. PolIV generates transcripts that are copied by RNA-dependent RNA polymerase 2 (RDR2) into dsRNA, which is processed by DICER-LIKE 3 (DCL3) to produce 24 nt heterochromatic siRNAs. The templates for PolIV-mediated transcription remain unknown; however, both methylated DNA and dsRNA have been proposed (Daxinger et al., 2009). PolV is not essential for siRNA biogenesis, but can enhance the accumulation of siRNA from specific genomic loci in Arabidopsis (Mosher et al., 2008). In the most accepted model of RdDM, PolV synthesizes nascent transcripts from genomic loci which have been modified by the SNF-type chromatin-remodeling protein, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1). The siRNAs associated with ARGONAUTE 4 (AGO4) interact with other downstream components such as PolV and SUPPRESSOR OF TY INSERTION 5 (SPT5) to guide de novo DNA methylation and chromatin silencing (Wierzbicki et al., 2008). Therefore, PolIV functions upstream of RdDM to produce and amplify the small-RNA trigger for silencing, whereas PolV acts downstream to transcribe non-coding RNAs and generate the scaffolds which attract silencing complexes. These scaffolds may also be involved in the reinforcement of silencing by serving as template for RDR2, through a positive-feedback loop. Several independent studies have demonstrated that PolIV and PolV share many small subunits with Pol II, and the remaining subunits are functionally diversified variants of PolII counterparts (He et al., 2009; Huang et al., 2009; Ream et al., 2009). It is likely that the different functions of PolIV and PolV are determined by the extended carboxy-terminal domain of the largest PolV subunit, which contains neighboring tryptophan–glycine/glycine–tryptophan residues (WG/GW repeats) that specifically interact with AGO4 (El-Shami et al., 2007), a downstream effector protein of the RdDM pathway that binds siRNAs. The role of AGO4 in
plant defense will be investigated in this thesis; therefore, ARGONAUTE proteins will be discussed in more detail in the next section.

1.5.2 Arabidopsis ARGONAUTE proteins

ARGONAUTE proteins are roughly 100 kDa in size, and contain two conserved domains: a 10 amino acid (aa) N-terminal PAZ domain and a 300 aa C-terminal PIWI domain (Carmell et al. 2002). The PAZ domain is thought to be required for protein-protein interactions, potentially mediating either heterodimerization or homodimerization. PAZ domains are also present in DCL proteins and have been shown to bind to the ends of small RNAs (Kidner and Martienssen, 2005). The PIWI domain is required for the cleavage of miRNA-targeted mRNA and is highly conserved in other eukaryotes (Carmell et al., 2002; Kidner and Martienssen, 2005).

The ARGONAUTE family is comprised of 10 genes in *A. thaliana*. The 10 ARGONAUTE proteins in *Arabidopsis* are phylogenetically classified into 3 clades: the first contains AGO1, AGO5 and AGO10; the second contains AGO2, AGO3 and AGO7 and the third contains AGO4, AGO6, AGO8 and AGO9 (Vaucheret, 2008). The *Arabidopsis* AGO1 (ARGONAUTE1), AGO4, AGO7 (ZIPPY) and AGO10 (PINHEAD/ZWILLE) genes have been extensively characterized (Moussian et al., 1998; Carmell et al., 2002; Kidner and Martienssen, 2005). The phenotypes of ago1 and ago10 mutants are associated with a loss of stem cell maintenance and auxilliary meristem failure, with both proteins having some degree of functional redundancy. It has also been shown that AGO10 associates with miR172 and miR165/166 and regulate the termination of floral stem cells (Ji et al., 2011). AGO1 is expressed throughout the plant at all stages of development and ago1 mutants exhibit abnormalities including radialized leaves, infertile
flowers and filamentous structures resembling the tentacles of a squid, the likely inspiration for naming this mutant *Argonaute* (Carmell et al., 2002). AGO1 has been referred to as an RNA ‘slicer’ and is required for the proper functioning of mi/siRNAs (Baumberger and Baulcombe, 2005). It appears to be the only argonaute protein required for post-translational gene silencing (PTGS) in plants. However, due to the apparent redundancy and higher levels of AGO1 expression throughout the plant compared to AGO10, it is not known whether AGO1 expression masks the role played by AGO10 (Lynn et al., 1999; Kidner and Martienssen, 2005).

1.5.3 *Arabidopsis* ARGONAUTE 4 – A key downstream component of the RdDM pathway

AGO4 is the most well characterized and functionally important member of the third *Arabidopsis* ARGONAUTE clade. AGO4 is a key component of the RdDM pathway and binds 24 nt siRNAs to direct cytosine methylation. AGO4 co-localizes with the RdDM factors PolIV, RDR2 and DCL3 to nuclear Cajal-bodies (Li et al., 2006), or with other components of the RdDM pathway, such as PolV and DRM2, to nuclear AB-bodies (Li et al., 2008). In *Arabidopsis*, *ago4* mutant plants produce fewer leaves, have a shorter bolting time (Liu et al., 2004), and have enhanced disease susceptibility compared to wild-type plants to a compatible *Pseudomonas syringae* strain (Agorio and Vera, 2007), suggesting that AGO4 plays a role in antibacterial defense. AGO4 is also implicated in virus-induced gene silencing (VIGS) in plants (Jones et al., 2006), as *Nicotiana benthamiana* plants with suppressed AGO4 expression have less efficient VIGS than wild-type plants.
1.5.4 DNA Methylation

Cytosine methylation was discovered in the early 20th century, and is an evolutionarily conserved DNA modification which occurs in both eukaryotes and prokaryotes (Gehring and Henikoff, 2007). The main function of DNA methylation in prokaryotes is to protect host cells from foreign DNA-like bacteriophages; whereas DNA methylation plays more diverse functions in eukaryotes (Goll and Bestor, 2005). In mammals and plants, DNA methylation is associated with genomic imprinting (Reik et al., 2001; Bird, 2002; Chan et al., 2005; Gehring and Henikoff, 2007). One of the major functions of DNA methylation is to protect the genome from both pathogenic and parasitic invaders such as viruses, transposable elements and transgenes. Methylation of the promoters of various genomic repeat elements and transposable elements prevents their transcription, in a mechanism referred to as transcriptional gene silencing (TGS) (Chan et al., 2005; Gehring and Henikoff, 2007; Zilberman et al., 2007). The regulation of gene expression by DNA methylation is facilitated by the formation of transcriptionally repressive heterochromatin structures (Chan et al., 2005; Hsieh and Fischer, 2005), which are tightly packed and transcriptionally inert as access to the transcriptional machinery is restricted (Lippman and Martienssen, 2004; Hsieh and Fischer, 2005). Loss of methylation leads to the activation of genes which were previously repressed by methylation (Gehring and Henikoff, 2007). Chromatin is composed of various histone proteins, including H2A, H2B, H3 and H4, and DNA methylation in Arabidopsis coincides with the methylation of H3K9 and K27 at heterochromatic loci (Hsieh and Fischer, 2005; Martin and Zhang, 2005).

DNA methylation is confined to CG in animals. In contrast, DNA methylation occurs in all contexts in plants, including CG, CNG and CNN (N = A, C, or T), although CG methylation is
predominant. In *Arabidopsis*, the overall rates of CG, CNG and CNN methylation are 24%, 6.7% and 1.7%, respectively (Cokus et al., 2008). Each methylation landscape is established and maintained by DNA methyltransferase enzymes (Bender, 2004; Lippman and Martienssen, 2004; Chan et al., 2005). The RdDM pathway can lead to the methylation of cytosines in all sequence contexts (Mathieu et al., 2007). Methylation in the CG context is maintained by METHYLTRANSFERASE 1 (MET1) and CNG methylation is maintained by the methyltransferase CMT3 (Aufstaz et al., 2004; Bartee et al., 2001) whereas cytosine methylation at CNN sites depends entirely on *de novo* methylation by the RdDM pathway, which is not maintained in the absence of 24 nt siRNAs (Cao and Jacobsen, 2002). Extensive DNA methylation is commonly observed at repeats, transposons and centromeric regions in *Arabidopsis* (Zhang et al., 2006; Zilberman et al., 2007) and a large proportion of *Arabidopsis* genes (20-33%) are also methylated (Tran et al., 2005; Zilberman et al., 2007). Transposons are heavily methylated along their lengths; whereas gene methylation is distributed within gene bodies away from 5’ and 3’ ends (Zhang et al., 2006; Zilberman et al., 2007). The general absence of methylation at the 5’ and 3’ ends of genes indicates that these regions must be unmethylated for efficient transcription to occur (Gehring and Henikoff, 2007; Zilberman et al., 2007). The unique patterns of DNA methylation at various loci, including transposons and endogenous genes, reflect the fact that DNA methylation is a highly regulated process.

1.5.5 DNA demethylation

DNA demethylation is observed in plants and mammals and can occur actively or passively. DNA can be passively demethylated due to the inhibition of maintenance methyltransferases during DNA replication, which allows methylated cytosines to be pruned and become
unmethylated (Goll and Bestor, 2005). Active demethylation requires DNA glycolases (DEMETER or DME and ROS1) which remove the methyl groups from cytosine residues, even in the absence of DNA replication (Agius et al., 2006). During mammalian embryogenesis, for example in mice, pigs and cows, the entire paternal genome undergoes demethylation soon after fertilization (Oswald et al., 2000; Bird, 2002; Santos et al., 2002). On the other hand, methylation of the maternal genome is maintained until the beginning of mitotic division, after which both the paternal and maternal genomes undergo passive demethylation (Abdalla et al., 2009). Active demethylation has also been observed in the vertebrate Xenopus laevis, where oct4, a gene which is expressed during gametogenesis and embryonic development, undergoes active demethylation indicating that demethylation plays an important role during the growth and development of both mammals and vertebrates (Simonsson and Gurdon, 2004; Barreto et al., 2007).

1.5.6 Possible mechanisms of DNA demethylation

Two mechanisms of DNA repair have been proposed: base excision repair (BER) and nucleotide excision repair (NER) (Zhu et al., 2000). BER is initiated by DNA glycolases which recognize lesions and excise damaged bases by cleavage of the N glycosidic bond between a 5’-methyl cytosine (5’-meC) base and deoxyribose, creating an abasic site (apurinic/apyrimidinic; AP) site. Subsequently, AP endonucleases remove the deoxyribose at AP sites and the gap is filled by DNA polymerase and ligase, resulting in the replacement of methylated cytosines with unmethylated cytosines (Jost et al., 1995; Jost et al., 1999; Zhu, 2009).

NER involves the direct excision of methyl groups by hydrolysis, resulting in replacement of a methyl moiety by a hydrogen atom and the release of methanol. Methyl CPG-binding domain
protein (MBD2) was the first protein reported to demethylate DNA by breaking the C-C bond (Bhattacharya et al., 1999) however, this finding could not be reproduced by the others (Ng et al., 1999). Active demethylation can also occur via the coupled activity of 5’-meC deaminase, which converts 5’-MeC to T, and G/T mismatch DNA glycolases such as thymine DNA glycosylase (TGD) which repair G/T mismatches (Morgan et al., 2004).

### 1.5.7 DNA demethylation in plants

A forward genetic screen of *Arabidopsis* led to the discovery of DNA glycolases which repress DNA methylation, resulting in gene activation. DNA demethylation in *Arabidopsis* is performed by the DEMETER (DME) family of DNA glycolases which contains 4 members: DME, REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2) and DML3 (Choi et al., 2002; Agius et al., 2006; Gehring et al., 2006; Gehring and Henikoff, 2007). DME is implicated in genomic imprinting which regulates the differential expression of parental alleles; whereas ROS1 demethylates transgenes and endogenous genes to maintain their expression (Choi et al., 2002; Agius et al., 2006). The function of DML2 and DML3 are not yet known.

### 1.5.8 Demethylation by ROS1

The role of ROS1 was revealed when a RD29A promoter-driven luciferase transgene (RD29A::LUC), which is subject to AGO4- or AGO6-catalysed RNA-directed DNA methylation, was observed to be hypermethylated and transcriptionally repressed in a *ros1* mutant (Gong et al., 2002; Agius et al., 2006; Morales-Ruiz et al., 2006; Zheng et al., 2007). This indicated that ROS1 demethylated DNA to maintain the expression of transgenes (Agius et al.,
2006; Morales-Ruiz et al., 2006). In vitro experiments confirmed that ROS1 encodes a 5’-meC DNA glycosylase. DNA REPLICATION PROTEIN 2 (RPA2) has also been shown to be involved in DNA repair and is conserved from yeast to mammals (Xia et al., 2006). ROS1 physically interacts with RPA2, and ros1/rpa2 double mutant plants are hypersensitive to methyl methanesulfonate (MMS), a genotoxic agent involved in DNA breakage, compared to ros1 or wild-type plants (Xia et al., 2006).

1.5.9 DNA demethylation by DEMETER

DEMETER activity is required for seed viability in Arabidopsis. DME is expressed in the central cells and synergids of female gametophytes, and leads to the specific removal of DNA methylation markers on the maternal alleles of genes such as MEDEA (MEA), FLOWERING WAGENINGEN (FWA) and FERTILIZATION INDEPENDENT SEED2 (FIS2) (Choi et al., 2002; Kinoshita et al., 2004; Jullien et al., 2006; Penterman et al., 2007). Paternal alleles are silenced by DNA methylation, as DME is not expressed in sperm cells. Seeds which inherit a maternal dme allele abort regardless of the paternal allele, as MEA, FWA and FIS2, which are all required for embryo development, fail to be expressed (Choi et al., 2002; Kinoshita et al., 2004; Jullien et al., 2006; Penterman et al., 2007). MEA, FIS2 and FWA are silenced due to CG methylation which is maintained by METHYLTRANSFERASE 1 (MET1) (Soppe et al., 2000; Kankel et al., 2003; Kinoshita et al., 2004; Penterman et al., 2007). Suppressed seed abortion is also observed in met1 mutant plants, suggesting a role for DME in the positive maintenance of MEA, FIS2 and FWA expression (Xiao et al., 2003). It has also been demonstrated that the maternal allele of MEA is hypomethylated relative to the non-expressed paternal allele, further suggesting that DME is required for maternal allele specific hypomethylation (Gehring et al., 2006). Maternal
allele-specific expression of \textit{FWA} in the endosperm is also dependent on DME (Kinoshita et al., 2004). \textit{In vitro} experiments have demonstrated that the purified DME DNA glycosylase can excise 5’-meC in the CG, CNG and CNN contexts, and this activity is responsible for the transcriptional activation of \textit{MEA}, \textit{FIS2} and \textit{FWA} (Gehring and Henikoff, 2007; Penterman et al., 2007).

\textbf{1.5.10 Regulation of demethylase gene expression}

As described earlier, DNA methylation is a highly controlled process; therefore, the expression of demethylase genes must also be tightly controlled (Zhu, 2009). ROS1, DME and DML2/3 are differentially expressed in \textit{Arabidopsis}. For instance, DME is mainly expressed in the reproductive organs; whereas ROS1 and DLM2/3 are expressed widely in plants (Choi et al., 2002; Gong et al., 2002). In mammals, demethylation mainly occurs at certain developmental stages and the demethylation of certain loci is subjected to developmental and environmental controls (Zhu, 2009).

Methylation and demethylation pathways appear to be closely linked. Expression of ROS1 is dramatically reduced in \textit{maintenance of DNA methylation (met1)} mutant plants. Similarly, mutants of the factors required for RdDM, such as \textit{rdr2}, \textit{dcl3}, \textit{drm2}, \textit{ago6}, \textit{polIV} and \textit{polV}, express lower levels of ROS1 (Huettel et al., 2006; Mathieu et al., 2007) and locus-specific methylation is lost; however, the overall level of DNA methylation is not severely affected (Huettel et al., 2006; Zheng et al., 2008). This suggests that the methylation levels of certain loci can be sensed, and that the expression of ROS1 is regulated accordingly (Zhu, 2009). ROS3 is a demethylation factor which is also regulated by DNA methylation. ROS3 is an RNA-binding
protein required for demethylation at some loci targeted by ROS1. Similarly to ROS1, ROS3 is dramatically downregulated in the ago6 mutant (Zheng et al., 2008). Interestingly, ROS1 is upregulated in ros3 mutant plants, and ROS3 is upregulated in ros1 mutant plants (Zheng et al., 2008). The increased methylation of a number of loci in ros1 or ros3 mutants is associated with increased expression of active demethylation factors such as ROS1 or ROS3 (Zheng et al., 2008), and it has been suggested that the expression levels of the entire range of demethylation factors is responsive to DNA methylation (Zhu, 2009).

1.5.11 Targets of the RdDM pathway

Many sequences targeted by factors associated with the RdDM pathway have been identified in the Arabidopsis genome using various molecular techniques. Techniques such as suppression subtractive hybridization (SSH) and amplified fragment length polymorphism (AFLP) analysis have been used to identify differential accumulation of transcripts in drd1 mutant and wild-type plants (Huettel et al., 2006). In the drd1 mutant, genes involved in metabolism, photosynthesis and protein synthesis were differentially expressed compared to wild-type plants (Huettel et al., 2006). Using a genome tilling array technique, 215 genes and hundreds of intergenic noncoding RNAs were identified to be differentially expressed in the ddc (drm1/drm2/cmt3) triple mutant compared to wild-type (Zhang et al., 2006). Similarly, differential expression of hundreds of genes was reported in an rdr2 mutant relative to wild-type (Kurihara et al., 2008). In Chapter 4, the differential expression of genes in RdDM and DNA demethylation mutants will be determined using microarray techniques, and the potential roles of these genes in plant defense will be discussed.
1.6 RNA silencing and plant defense against pathogens

1.6.1 RNA silencing and virus resistance in plants

Viruses are intracellular pathogens which infect all forms of life. Viral genomes are comprised of either single or double-stranded RNA or DNA and packaged into virions. Most viruses replicate in host cells using RNA-dependent RNA polymerases, DNA replicases or reverse transcriptases encoded by their own genome; however, some viral pathogens, such as viroids, use host-encoded RNA polymerases to replicate.

Viruses are both inducers and targets of RNA silencing. Virus-mediated silencing can occur with RNA viruses which replicate in the cytoplasm, and also with DNA viruses which replicate in the nucleus (Voinnet, 2005). Transgenes which constitutively express part of the viral genome can induce resistance to infection by the same virus in plants (Marathe et al., 2000). RNA silencing, as an antiviral defense mechanism, is best illustrated by the processes of natural host recovery from viral infections and viral cross protection. For example, when *Brassica napus* is infected with cauliflower mosaic virus, the symptoms progressively increase up to 30 to 40 days postinoculation, and then decline thereafter as the plants recover. At 50 days post inoculation, newly emergent leaves remain asymptomatic (Covey et al., 1997). In viral cross protection, the prior infection of plants with a milder virus confers resistance to subsequent infection with severe strains of related viruses (Covey et al., 1997; Ratcliffe et al., 1999). Both natural host recovery and cross-protection are mediated by RNA silencing (Voinnet, 2005).

Replication of all types of viruses and subviral agents is associated with the accumulation of siRNAs in plants. Accumulation of siRNAs has not been detected in virus-infected mammalian
cells; however, some mammalian viruses encode miRNAs (Sarnow et al., 2006). RNA viruses predominantly produce 21 and 22 nt siRNAs in plants; however, all three size classes of siRNAs, including 24 nt siRNAs, are associated with DNA viruses (Blevins et al., 2006; Fusaro et al., 2006; Moissiard and Voinnet, 2006; Ding and Voinnet, 2007), indicating that viral RNAs can be targeted by all species of siRNA. It is possible that siRNA-guided RNA cleavage may not be the only mechanism by which RNA silencing inhibits viral replication. The genomes of DNA viruses are targeted by siRNA-directed de novo DNA methylation, and this RdDM can lead to the transcriptional silencing of viral genes and hence inhibit viral replication. In addition to cis-acting virus-derived siRNAs, host-encoded miRNAs and siRNAs can also target viral RNAs and therefore contribute to viral defense. Host-encoded miRNAs have been shown to play a critical role in antiviral resistance in mammalian cells (Sarnow et al., 2006); however, evidence for a similar mechanism is still lacking in plants. For instance, human-encoded miRNAs have been shown to target human immunodeficiency virus (HIV) genes to suppress HIV replication. In turn, HIV actively suppresses the expression of host antiviral miRNAs, in order to maintain its replication (Kumar, 2007). Similarly, a cellular miRNA, miR-32, can effectively restrict accumulation of the retrovirus primate foamy virus (PFV-1) in human cells, and conversely the Tas protein encoded by PFV-1 suppresses the function of the cellular miRNA (Lecellier et al., 2005). The involvement of host small RNAs in antiviral defense may have played a key role in evolution of the viral genome. Sequence variations in the viral genome, which prevent viruses from being effectively targeted by specific host-encoded small RNAs, may be positively selected during viral genome evolution, and such a mechanism of small RNA-mediated selection may also account for some viral host specificities (Wang et al., 2004).
Negative regulation of gene expression may not be the only function of small RNAs during viral replication. A human liver-specific miRNA can positively regulate replication of the hepatitis C virus (Jopling et al., 2005), and it is possible that similar small RNA-mediated mechanisms may exist for plant viruses.

Viruses have evolved a number of methods to overcome the RNA silencing-mediated host defense mechanism (Ding and Voinnet, 2007). Almost all plant viruses encode RNA silencing suppressors (Table 2), most of which bind to long dsRNA or siRNA duplexes, to prevent the production of siRNA or loading of siRNA to the RISC. For instance, the viral suppressor protein p19 from the plant tombusvirus specifically binds duplex 21 nt siRNAs, preventing formation of RISC (Lakatos et al., 2004). Similarly, the potyvirus P1/HC-Pro suppressor prevents unwinding of the siRNA duplex and inhibits loading of siRNA into the RISC (Lakatos et al., 2006). Some silencing suppressors, such as potexvirus p25, inhibit the spread of systemic silencing signals (Lakatos et al., 2006). A recent study suggested a novel suppressor mechanism, by which polerovirus-encoded F-box protein (P0) targets the PAZ motif and the adjacent upstream sequence of AGO1, to mediate AGO1 degradation and suppress RNA silencing (Baumberger et al., 2007). Animal viruses have also been shown to encode RNA silencing suppressors (Li and Ding, 2005).

In plants, DNA viruses appear to be more resistant to RNA silencing than RNA viruses (Wang et al., 2006), presumably due to their DNA-based genome. DNA viruses have also been shown to encode silencing suppressor proteins (Bisaro, 2006), which may further inhibit silencing by minimizing siRNA-mediated degradation of viral RNAs. Small subviral RNAs which do not
encode proteins have evolved a unique mechanism to combat RNA silencing, as the genomic RNAs of viroids and satellite RNAs forms stable secondary structures which are resistant to RISC-mediated cleavage (Wang et al., 2004; Itaya et al., 2007). A recent study demonstrated that methylation-deficient Arabidopsis mutants are hypersusceptible to geminiviruses, and also that RNA-directed DNA methylation pathway components such as AGO4 are necessary for host recovery from infection. Geminivirus DNA and the associated histones are methylated in infected plants, and viral DNA methylation is reduced in mutants that display enhanced disease (Raja et al., 2008).

1.6.2 RNA silencing in plant defense against non-viral pathogens and pests

Recent studies have provided evidence that both siRNA and miRNA pathways play a role in plant defense against bacteria, fungi and insects, as well as viruses. The involvement of siRNAs in plant defense against non-viral pathogens was first observed with Agrobacterium tumefaciens (Dunoyer et al., 2006). SiRNAs corresponding to T-DNA oncogenes accumulate in A. tumefaciens-infected plant tissues, and RNA silencing-deficient plants (rdr6 mutant and transgenic plants expressing the p38 silencing suppressor) are hypersusceptible to A. tumefaciens. Successful A. tumefaciens infection relies on the establishment of an anti-silencing state in tumors, by specifically inhibiting production of siRNAs by DICER proteins (Dunoyer et al., 2006). The similarity of this process to plant-viral interactions suggests that plant-bacterial interactions may use the same pathways.

Several recent reports have also indicated the involvement of siRNAs in plant resistance against the bacterial pathogen P. syringae. As discussed earlier, a natural antisense siRNA (nat-
siRNAATGB2) is strongly induced in Arabidopsis upon infection by P. syringae pathovar tomato (Pst), and nat-siRNAATGB2 downregulates a PPRL gene which encodes a negative regulator of the RPS2 disease resistance pathway. As a result, induction of nat-siRNAATGB2 increases RPS2-mediated race-specific resistance against Pst in Arabidopsis (Katiyar-Agarwal et al., 2006). Recently a new class of 30-40 nt small RNAs, known as long siRNAs (lsiRNAs), were found to be induced by P. syringae (Katiyar-Agarwal et al., 2007). Biogenesis of one lsiRNA, AtlsiRNA-1, requires DCL1, DCL4, HYL1, HST1, HEN1, RDR6, PolIV and AGO7 (Katiyar-Agarwal et al., 2007), and AtlsiRNA-1 contributes to plant bacterial resistance by silencing AtRAP, which is a negative regulator of plant defense (Katiyar-Agarwal et al., 2007).

The miRNA pathway is also involved in plant interactions with both A. tumefaciens and P. syringae. The effect of miRNAs on A. tumefaciens infection appears to be the opposite of siRNAs: siRNAs appear to restrict A. tumefaciens infection; whereas the processing and activities of endogenous miRNAs appear to be indispensable for efficient A. tumefaciens infection. Loss-of-function mutations in DCL1 and HEN1, factors required for miRNA biogenesis, almost completely inhibit A. tumefaciens infection of Arabidopsis (Dunoyer et al., 2006). Overexpression of viral-encoded silencing suppressors which inhibit miRNA-guided functions has a similar suppressive effect on A. tumefaciens infection (Dunoyer et al., 2006). Additionally, a P. syringae bacterial flagellin-derived peptide has been observed to induce miR393 in Arabidopsis, which negatively regulates messenger RNAs expressing F-box auxin receptors, resulting in increased resistance to P. syringae (Navarro et al., 2006). Overexpression of miR393 reduces the plant bacterial titer by approximately five-fold (Navarro et al., 2006). A recent report demonstrated that specific microRNAs are associated with disease development.
induced by fusiform rust in pine, suggesting that the miRNA pathway may also be involved in plant interaction with fungal pathogens (Lu et al., 2007).

The involvement of small RNA pathways in plant defense against non-viral pathogens is further confirmed by the results of recent high-throughput sequencing of small RNAs in plants. For instance, deep sequencing of small RNAs in Arabidopsis and tomato frequently identified siRNAs and miRNAs corresponding to resistance genes or gene clusters with function against viral, bacterial, fungal and nematode diseases (Fahlgren, 2007). This suggests that some disease resistance genes may be negatively regulated by small RNA pathways, either post-transcriptionally via mRNA degradation or transcriptionally by DNA methylation. Notable examples of miRNAs with the potential to negatively regulate disease genes include miR472 and miR772, which have been confirmed to target several nucleotide-binding site–leucine-rich repeat (NBS-LRR) disease resistance genes in Arabidopsis (Rajagopalan et al., 2006; Howell et al., 2007). It is possible that the suppression of disease resistance genes by siRNAs and miRNAs is important for normal plant development, as excessive accumulation of these products may have deleterious effects on plant cells. Pathogen infections may lead to altered dysregulation of these siRNAs and miRNAs, inducing increased expression of disease resistance genes and enhanced disease resistance. In addition to bacteria, one study demonstrated that silencing of the RNA-dependent RNA polymerase gene (RDR1) in Nicotiana attenuata, either with a virus-induced silencing vector or with an i/r transgene, significantly increased the susceptibility to attack by herbivorous insects such as Manduca sexta, mirids, beetles and grasshoppers (Pandey and Baldwin, 2007), indicating that siRNAs may play a role in plant defense against insect pests, as RDRs are an important component of siRNA biogenesis.
1.6.3 Critical role of downstream factors of RdDM pathway in response to fungal and bacterial pathogens in *Arabidopsis*

The Arabidopsis *ocp* (for overexpressor of cationic peroxidase) mutants were identified in a genetic screen designed to isolate negative regulators of pathogen-induced defense responses (Coego et al., 2005). Subsequently, the same laboratory has characterized an *ocp11* mutant that overexpresses the H$_2$O$_2$-responsive Ep5C promoter fused to the β-glucuronidase reporter gene. The *ocp11* mutant exhibits enhanced disease susceptibility to several virulent and avirulent strains of the bacterial pathogen *P. syringae*. *OCP11* was cloned and found to encode AGO4, a downstream component of the RdDM pathway. Another mutant allele, *ago4-1*, was examined and likewise found to be compromised in resistance to *P. syringae*. AGO4 was found to function independently of other components of the RdDM pathway in conferring resistance to *P. syringae*. This report indicated that RdDM pathway, or at least the component of RdDM pathway, AGO4, is involved in resistance to bacterial pathogens (Agorio and Vera, 2007).

Recently, in a genetic screen aimed to identify and characterize the contribution of other components of the RdDM pathway in plant immunity, identified *ocp1*, a recessive mutant allele of NRPD2, a second largest subunit shared between PolIV and PolV. *ocp1* along with other RdDM pathway mutants such as *nrpd1, nrpe1, ago4, drd1, rdr2, drm1 drm2* and *nrpd1 nrpe1* double mutants were screened for altered resistance or susceptibility in response to fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. The screening analysis indicated that PolV, but not PolIV, was required for pathogen defense against fungal infection in *Arabidopsis*, as *nrpd1 nrpe1* (double mutant defective in both Pol IV and Pol V activities) and *nrpe1* plants had a higher susceptibility to fungal infection than the *nrpd1* (PolIV) mutant and wild-type plants (López et al., 2011).
The Jasmonic acid (JA)-responsive genes are mainly involved in fungal defense against necrotrophic pathogens such as *Botrytis cinerea* and *Plectosphaerella cucumerina* (López et al., 2011; Bari and Jones, 2009). The response of the PLANT DEFENSIN 1.2A (*PDF-1.2a*) gene, a molecular marker of an intact jasmonic acid pathway, was significantly attenuated in *P. cucumerina*-infected *polIV* mutants compared to wild-type plants (López et al. 2011). The susceptible phenotype of *polIV* mutants in response to *P. cucumerina* infection is in agreement with the observation that *PDF1.2a* was down regulated (López et al. 2011).

In contrast to this, the expression of PATHOGENESIS RELATED GENE 1 (PR1), a molecular marker of an intact salicylic acid pathway (SA), was significantly up regulated in the *polV* mutants compared to the wild-type plants at 2 days following *P. cucumerina* infection. Interestingly, *polV* mutants were found to be more resistant to biotrophic bacterial pathogen *P. syringae* compared to the wild-type plants when screened for altered resistance or susceptibility to this pathogen. This is consistent with the role of SA pathway in imparting disease resistance against biotrophs.

These results highlight the importance of epigenetic control as an additional layer of complexity in the regulation of plant immunity and point towards multiple components of the RdDM pathway being involved in plant immunity based on genetic evidence, but whether this is a direct or indirect effect on disease-related genes is unclear (López et al. 2011).
1.6.4 Role of epigenetic modifications by histone acetyltransferases and histone deacetylases in plant defense

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the acetylation of histone lysine residues. Acetylated genes are transcriptionally active (Berger, 2007; Pfluger and Wagner, 2007); whereas histone deacetylation represses transcription (Wu et al., 2000; Tian et al., 2005; Tanaka et al., 2008), though some HDACs have been linked to transcriptional activation (Wang et al., 2002 and 2009; Zupkovitz et al., 2006). HATs and HDACs also play a role in plant pathogen defense. PolV suppresses the SA-pathway through acetylating SA-gene promoters via the RdDM pathway (López et al., 2011); however, the HDACs involved in this process have not yet been identified. In addition, maize corn leaf disease is caused by the Cochliobolus (Helminthosporium) carbonum (HC)-toxin, which inhibits a specific class of HDACs, including Reduced Potassium Dependency protein 3/Histone Deacetylase 1 (RPD3/HDA1) and HD2 (Brosch et al., 1995; Chen and Tian, 2007; Walton, 2006; Yang and Seto, 2008). The maize Hml/2 allele expresses a carbonyl reductase which can inactivate the HC-toxin (Johal and Briggs, 1992) and HDACs targeted by the HC-toxin induce altered histone acetylation in susceptible maize plants (Brosch et al., 1995; Ransom and Walton, 1997). HDACs are also inhibited by the Alternaria brassicicola fungal derivative depudecin in Arabidopsis (Wight et al., 2009). In the genetically tractable Arabidopsis thaliana – Alternaria brassicicola pathosystem, plant HDACs are inhibited by the fungal derivative depudecin. However, depudecin was shown not to be an important virulence factor (Wight et al., 2009).

The JA pathway provides defense against necrotrophic pathogens (Bari and Jones, 2009) and the ability of HDACs to activate the JA pathway has been well characterized. For example, AtHDAC19 (RPD3/HDA1) is upregulated in Alternaria brassicicola-infected plants. Silencing of
*AtHDAC19* reduces plant fungal susceptibility and leads to increased histone acetylation in the promoter regions of JA-pathway genes (Fong et al., 2006; Zhou et al., 2005). It is thought that HAC19 may induce fungal resistance by being recruited to the promoters of JA-responsive genes by Ethylene Responsive Factor (ERF) (Zhou et al., 2005), in a similar manner to the hormone-responsive mechanisms which regulate *Arabidopsis* stress-related genes (Song and Galbraith, 2006). HDAC6, another member of the RPD3/HDA1 family, regulates DNA methylation, transgene silencing and rRNA gene activity in *Arabidopsis* (Aufsatz et al., 2007). HDAC6 has also been linked to defense, as its interaction with the F-box protein CORONATINE INSENSITIVE 1 (COI1) is required for the induction of JA signaling (Devoto et al., 2002; Wu et al., 2008). Though the role of HDACs in JA pathway-dependent responses is relatively well characterized, the precise mechanisms and most substrates of HDACs during plant defense have not yet been identified (Alvarez et al. 2010).

In addition, other factors responsible for chromatin modifications have been linked to plant defense, mainly through the SA pathway, and are well reviewed by Alvarez et al. 2010. For example, HDAC19 has been linked to plant defense against *P. syringae*, and it interacts with the transcription factors WRKY38 and WRKY62 which suppress the SA pathway (Kim et al., 2008). The putative methyltransferase ARABDOPSIS TRITHORAX activates the SA-pathway to confer resistance to *P. syringae* (Álvarez-Venegas et al. 2006; 2007). In contrast, other members of the Swr-1-like complex, the SUCROSE NONFERMENTING 2 (Snf-2)-like protein BRAHMA (BRM) and the putative chromatin remodeler SUPPRESSOR OF NPR1, INDUCIBLE 1 (SNI1) have been linked to constitutive repression of the SA-pathway (Bezhani et al. 2007; Durrant et al. 2007; Mosher et al. 2007). Additionally, the Snf-2-like protein
SPLAYED (SYD), one of the SWI/SNF class of chromatin remodeling ATPases, is required for the resistance against *B. cinerea* in *Arabidopsis* (Walley et al., 2008).

### 1.6.5 *Arabidopsis* – *Fusarium oxysporum* interaction

Most studies of plant defense against fungal pathogens have focused on leaf-infecting pathogens; however, plant resistance mechanisms against root-infecting pathogens are poorly understood. There have been no previous studies reporting the involvement of RNA silencing in plant defense against *F. oxysporum*. This lack of understanding poses a significant barrier to the development of plant varieties which are resistant to root infecting pathogens. *F. oxysporum* is a root infecting pathogen which causes vascular wilt disease in more than 100 plant species, including economically important cotton, tomato and banana (Okubara and Paulitz, 2005). The infection process is best understood in tomato (Rep et al., 2002; Di Pietro et al., 2003; Berrocal-Lobo and Molina, 2008), where following germination of the spores in soil, the fungal hyphae penetrate the root tips and advance intercellularly to the root cortex, finally reaching the xylem vessels. From this point, the pathogen moves upwards through vascular tissue towards the stem. The vascular tissues become clogged as a result of fungal spore accumulation and compounds, such as tyloses, produced by the plant in response to *F. oxysporum*. Effectors or toxins produced by *F. oxysporum* are secreted into the conducting tissue or vessels, and carried to the shoot tissue where they lead to the characteristic *F. oxysporum* disease phenotype of vein chlorosis in the leaves (Di Pietro et al., 2003; Dombrecht et al., 2006; Berrocal-Lobo and Molina, 2008). *F. oxysporum* is considered a hemibiotroph, a pathogen whose life-cycle is partly on living plant tissue as a biotroph and partly on dead tissue as a necrotroph (Agrios, 2005).
*F. oxysporum* can infect the model plant *Arabidopsis*, making *Arabidopsis-F. oxysporum* an excellent pathosystem for this study. Another advantage of using *Arabidopsis* for this study is the wide range of genomic tools and genetic resources which are available for the study of *Arabidopsis* (Edgar et al., 2006). Furthermore, our laboratory has a large collection of *Arabidopsis* RNA silencing and DNA demethylation mutants, enabling a screen of various silencing-deficient mutants for altered responses to pathogens.
1.7 Objectives

The goals of this thesis are to:

- **Identify RNA silencing pathways/factors with a potential role in fungal disease resistance** - by screening Arabidopsis mutants deficient in various RNA silencing factors for susceptibility/resistance to *F. oxysporum*.

- **Characterize the role of the identified RNA silencing factors in resistance to fungal disease** – by examining the effect of these mutations on the expression of defense-related genes.
CHAPTER 2: Materials and Methods
2.1 Plant materials and growth conditions

Unless otherwise stated, Arabidopsis thaliana ecotype Colombia (Col-0) was used for all experiments. The seeds were sterilized for 3.5 h in a desiccator by contact with chlorine gas supplied from a mixture of 100 ml hypochlorite and 3 ml of 12 N HCl. Upon completion, the seeds were aseptically spread out onto 150 mm Murashige Skoog (MS) Noble agar plates (Appendix I), stratified for 48 h at 4°C, then transferred to a growth cabinet under long days (16 h light/8 h dark at 23°C). After germination, the plant seedlings were transferred to either soil or MS plates under long days (light: 27°C/16 h at 100 µmol/m²/sec; dark: 8 h/27°C).

2.2 Fusarium oxysporum inoculation protocol

The F. oxysporum strain used in this experiment was F. oxysporum BRIP 5176a (as listed in the Herbarium BRIP Accession Book Report; 16th of Feb, 1971 Det: Johnson, J.C., 19142, Johnson, J.C., Brassica oleracea, Indooroopilly, Brisbane, QLD, Australia) and provided to us by Dr. Kemal Kazan of CSIRO Plant industry, Brisbane. Fusarium oxysporum was maintained on sterile filter paper and stored at -80°C. When the fungal tissue was required, the dried filter paper containing F. oxysporum spores was placed onto agar plates (12g/L Agar) for 1 week. The Fusarium was then grown in 50mL flask of ½ strength PDA (24g/L of Potato Dextrose Broth (PDB). The flasks containing the inoculum were placed onto a shaker at 28°C for approximately 3 to 4 days. The broth is filtered through at least 4 layers of tissue papers and the spore solution diluted with sterile distilled water to a 1x10⁶ concentration or otherwise stated for inoculations, and was used to inoculate the plants.
2.3 Root inoculation in soil

The plants used for inoculation were grown in autoclaved soil for approximately 1-2 weeks prior to inoculation, carefully removed from the soil taking great care to preserve as much of the root system as possible, then the plant roots were rinsed in distilled water to remove extra soil. A small number of plants were replanted as mock-inoculated controls. The remaining plants were placed into inoculum of the desired spore concentration, and gently mixed for approximately 20 sec to ensure that the fungal spores did not settle to the bottom of the container and attached to the root to be carried into the new well-watered soil. The plants were placed into separate cells and limited watering was applied to maintain a high inoculum density in the soil around the root system. The plants were incubated at 28°C and *F. oxysporum*-induced wilt symptoms were scored approximately 5-7 days post-inoculation.

2.4 Root inoculation on sucrose-free MS plates

The inoculation method was developed by Dr. Tuan Le in our laboratory. Initially, the plants were gown on MS plates for approximately 3 weeks and carefully removed from the MS plates, taking great care to preserve as much of the root system as possible. The plant roots were dipped into distilled water for the mock controls, or *F. oxysporum* inoculum at the desired spore concentration, and then placed onto sucrose-free MS plates and incubated at 23-27°C with 16 h light.
2.5 Grafting

Arabidopsis seedling grafting was conducted according to the method of Turnbull et al. (2002). Briefly, Arabidopsis seeds were surface sterilized, plated on MS agar plates and stratified at 4°C. The plates were placed vertically in a 23°C growth room to allow the seedlings to grow along the surface of the medium in the same direction. Grafting was performed on 3-5 day-old seedlings by transferring the seedlings to a Petri dish containing one layer of 0.45 mm nitrocellulose filter paper (Millipore, Billerica, Massachusetts, USA) placed over two layers of Whatman No. 1 filter paper dampened with H₂O (Piscataway, New Jersey, City, ST, USA). Grafting was performed under a dissecting microscope using a No. 15 scalpel to make a transverse cut at the top of the hypocotyl, just below the cotyledons. The scions were placed on top of the rootstock, the surface water was allowed to evaporate; and then the plates were sealed with parafilm and incubated vertically under a long-daylight regime at 21°C. The grafts were assessed visually over 4–7 days, and successful grafts were strong enough to be lifted using the scion.

2.6 Fungal biomass estimation

DNA was extracted from the whole above-ground and below-ground tissues of plants inoculated with F. oxysporum using the DNeasy Plant Minikit. The levels of F. oxysporum and Arabidopsis DNA were determined by semi-quantitative and real-time quantitative PCR using specific primer-pairs for F. oxysporum glyceraldehyde 3-phosphate dehydrogenase (GPD) and cutinase, and Arabidopsis iASK (At5g26751; Gachon and Saindrenan, 2004). The relative fungal biomass was calculated by normalizing F. oxysporum GPD to Arabidopsis iASK.
2.7 *Agrobacterium* root inoculation assay

2.7.1 Agrobacterium tumefaciens strain

The oncogenic *A. tumefaciens* strain A281 carrying the pTiBo542 plasmid was used for the *Arabidopsis thaliana* root inoculation assay. *A. tumefaciens* was subcultured on Luria broth (LB) plates containing 100 µg/ml Rifampicilin for 1-2 days in an incubator at 30°C.

2.7.2 Root inoculation assay

Before inoculation, the colonies were washed in MS nutrients without agar using a sterile pipette, and the liquid was used for inoculation. Tumours were established on *A. thaliana* root segments by dissecting the root tissues using a scalpel and dipping the root segments in *A. tumefaciens* strain A281 bacterial cultures resuspended in MS nutrients without agar. Then, the individual root segments were transferred to MS plates under sterile conditions, incubated at 25°C for 2 days and then washed in sterile water to remove excess bacteria. The individual root segments were transferred onto MS media containing 100 µg ml\(^{-1}\) of the antibiotic timentin, cultured for 4 weeks at 25 °C, and the numbers of tumours were scored at different time points.

2.8 RNA extraction methods

2.8.1 TRIzol RNA extraction

Approximately 0.5-1 g plant tissue was collected for RNA extraction and ground to a fine powder under liquid nitrogen using a mortar and pestle. The samples were homogenised by the
addition of 5-10 ml TRIzol reagent (Invitrogen), transferred to a clean tube, incubated for 5 min at RT, 2 ml chloroform was added, the samples were shaken well, centrifuged at 7,000 g for 10 min at 4°C, the supernatant was removed to a clean tube, and the chloroform extraction step was repeated to remove all of precipitated proteins. After the second chloroform step, the aqueous phase was transferred to a clean tube, mixed with one volume of cold 100% isopropanol, incubated at -20°C overnight or -80°C for 2 h then centrifuged at 8,000 g for 20 min at 4°C. The supernatant was removed, the pellet was carefully washed with cold 80% ethanol, the RNA pellet was resuspended in DEPC-treated H₂O and the RNA concentration was determined using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, City, CA, USA).

2.8.2 Lithium chloride RNA extraction

Approximately 2 g plant tissue was ground into a fine powder under liquid nitrogen using a mortar and pestle, transferred to a pre-cooled 10 ml tube and stored dry until use. A solution of 2 ml RNA extraction buffer and 2 ml phenol (Sigma) was heated to boiling point and added to the samples. After thorough mixing by inversion, the samples were allowed to cool to RT, mixed with 2 ml chloroform, and centrifuged at 4,000 g for 15 min to separate the organic and aqueous phases. The aqueous phase was removed to a clean tube and the chloroform extraction was repeated to ensure protein was completely removed from the sample. A one-third volume of 8 M LiCl was added to the final supernatant, incubated overnight at 4°C, the samples were centrifuged at 4,000 g for 10 min; then the supernatant was transferred to a fresh 10 ml tube for isolation of small RNA and DNA. The pellet containing the large RNA fraction was washed with cold 80% EtOH and re-suspended in diethylpyrocarbonate (DEPC)-treated dH₂O. To isolate the
small RNA & DNA fraction, one volume of isopropanol was added to the supernatant from the previous step, incubated at RT for 4-6 h, centrifuged at 4,000 g for 20 min, washed with 80% EtOH, dried and then shaken overnight in 500 µl of 8 M LiCl at 4°C. The samples were centrifuged for 30 min at 4°C, the supernatant was collected and the 8M LiCl step was repeated. The remaining pellet was re-suspended for DNA analysis, whilst both supernatants were combined and small RNAs were precipitated using 1 volume of 100% isopropanol. After overnight incubation at -20°C, the samples were centrifuged at 18,000 g for 20 min at 4°C, the resultant pellet was washed several times with cold 80% EtOH to minimize the salt concentration, then the pellet was air-dried and resuspended in 100 µl DEPC dH₂O.

2.8.3 DNase treatment of total RNA

Approximately 30 μg total RNA was subjected to DNase-treatment in a 200 µl reaction volume containing 5 µl RQ1 RNase-free DNase (Promega, Madison, WI, USA), and 20 µl of 10 x RQ Buffer, at 37°C for 40 min, then the samples were incubated on ice for 10 min, purified using the RNeasy® Mini Kit (Qiagen) according to the manufacturer’s instructions, and resuspended in 50 µl RNase-free dH₂O.
2.9 PCR

2.9.1 Semi-quantitative PCR

Total RNA from the whole plant tissue (10-12 plants in each sample) of three week-old plants grown on MS agar plates was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany) following the recommended protocol with DNaseI treatment.

Five microlitres of purified DNase-treated total RNA was mixed with 1.0 μl of 50 μM oligo dT23, 2.0 μL of 5 mM dNTPs and dH2O to a total volume of 13 μl. The reaction mix was denatured at 65°C for 5 min, immediately chilled on ice for 5 min then 5 μl First-Strand Buffer, 1.0 μl of 0.1 M DTT, 1.0 μl SuperScript™ III (Invitrogen) and 1.0 μL RNasin® (Promega) were added and incubated at 50°C for 60 min for first strand cDNA synthesis. The reaction was terminated by incubation at 70°C for 20 min, 30 μl RNase-free dH2O was added to provide 50 μl cDNA. The cDNA concentration was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) and the cDNA quality was assessed by electrophoresis using an EtBr-stained 1.0% agarose gel.

Quantitative real-time PCR

Quantitative RT-PCR (qPCR) was performed on at least two biological replicates for each sample using the SYBR Green JumpStart Taq ReadyMix (Sigma); no template controls were performed in parallel. The qPCR reactions were performed on a Rotor-Gene 2000 Real-Time Cycler (Corbett Research, Sydney, NSW, Australia) for 4 min at 94°C; then 40 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C, followed by a melting-curve program (72°C–95°C with a 5 s hold at each temperature). Fluorescence data were acquired at the
72°C step during each amplification cycle and continuously during the melting-curve program. Comparative quantification analysis (Rotorgene-6 software, Corbett Research) was used to determine the relative amount of cDNA for the gene of interest for each sample. This method uses the start of the exponential phase of amplification (take-off point) and average reaction efficiency for each samples, enabling a direct comparison of different samples, to determine the relative level of expression in each sample. In each experiment, the cDNA concentration of the gene of interest in the experimental samples was compared to the cDNA concentration of the gene of interest in the appropriate control samples. Each primer set was used to amplify each cDNA template in triplicate, and the average relative concentration was determined from the three technical replicates. The expression levels of the control gene FORMALDEHYDE DEHYDROGENASE (FDH; At5g43940) were determined in a similar manner. Then, the data was normalized by determining the ratio of the concentration of the gene of interest to the concentration of the control gene. The reaction products were electrophoreosed on ethidium bromide (EtBr)-stained 1.0% agarose gels to confirm the size of the amplified PCR products. The experiments were conducted on at least two biological replicates with similar results.

2.10 RNA hybridisation

2.10.1 Probe construction

The AGO4 probe sequence was amplified from Arabidopsis Col-0 wild-type cDNA using the primers designed for qRT-PCR. The probe was 200–250 bp in length, and the PCR product of the expected sizes was ligated into the pGEM®-T Easy Vector (Promega), as described in
section 2.10. The plasmids were linearized using restriction endonuclease digestion (New England Biolabs, Ipswich, MA, USA) upstream of the 5′ end of the inserted sequence. The linearized fragments were used as templates for the synthesis of γ-32P-labelled antisense transcripts complementary to the target RNAs. Either the T7 or the SP6 transcription initiation sequences of pGEM®-T Easy were used for probe synthesis, depending on the orientation of the insert. The probes were transcribed by incubation with T7 or SP6 at 37ºC for 1 h, then 1.0 μl RNase-free DNase was added and incubated at 37ºC for 10 min. The probes were precipitated by adding 10 μl of 7.5 M NH₄OAc and 75 μl of 100% ethanol, incubated for 10 min on ice, centrifuged at 15,700 g for 15 min and the RNA pellet was resuspended in 100 μl TE buffer. Then, 50 μl of 7.5 M NH₄OAc and 375 μl of 100% ethanol were added, incubated on ice for 10 min, centrifuged at 15,700 g for 15 min, and the RNA pellet was air-dried for 5 min at RT and resuspended in 20 μl TE buffer.

2.10.2 Separation of RNA on formaldehyde agarose gels and transfer to membranes

Formaldehyde-based 1.4% agarose gels (100 ml) were prepared with 1.4 g agarose, 85 ml dH₂O; 10.0 ml of 10 x MOPS Buffer containing 200 mM MOPS (Sigma), 50 mM sodium acetate, 10 mM EDTA, pH 7.0. Five milliliters of deionised formaldehyde was added just prior to pouring the gel into standard gel electrophoresis apparatus. A 10 μL aliquot of each RNA sample (10 μg total RNA) was mixed with 10 μl formamide, 3.5 μl formaldehyde, 2.0 μl of 10 x MOPS buffer and 1.0 μl diluted EtBr. The RNA was denatured by heating at 65ºC for 5 min, then 5.0 μl of 10 x RNA loading buffer (3.0 g ficoll, 0.025 g Xylene Cyanol FF [XCFF], 0.025 g bromophenol blue [BPB] and 10.0 ml dH₂O) were added, and the sample was immediately loaded on the gel. The gels were run in 10x MOPS buffer until the lower dye front had migrated through three-
quarters of the gel. The gels were visualised using a UV transilluminator and an image was captured and saved for use as a loading control. HyBond-N+ membrane (GE Healthcare, Piscataway, NJ, USA) was presoaked in 20x SSC, placed on top of the formaldehyde gel and the separated RNA was blotted onto the HyBond-N+ membrane by capillary blotting overnight in 20x SSC. The blotted RNA membrane was placed on damp 3MM filter paper (Whatman) and UV cross-linked using the Stratalinker® UV Crosslinker (Stratagene, Sydney, Australia).

2.10.3 Pre-hybridisation and hybridisation

The membranes were pre-hybridised at 42°C for 2 h in 50 ml sRNA hybridisation buffer consisting of 50% formamide, 1.0% SDS, 5.0 x SSPE, pH 7.4 (3.0 M NaCl; 0.2 M NaH₂PO₄; 0.02 M EDTA) and 5.0 x Denhardt’s solution (2.0% Ficoll 400, 2.0% polyvinylpyrrolidone, 2.0% bovine serum albumin [BSA]). The end-labelled DNA oligonucleotides were directly added to the hybridisation buffer and hybridised to the membranes for approximately 16 h at 42°C.

2.10.4 Washing and visualisation

Following overnight hybridisation, the membranes were washed twice for 30 min in 2x saline-sodium citrate (SSC) buffer containing 0.2% Sodium Dodecyl Sulfate (SDS) at 42°C. The membrane was placed on wet 3MM (Whatman) paper, sealed in a plastic envelope, exposed in a phosphor screen overnight and imaged using the FLA-5000 imaging system (Fujifilm). U6 small nuclear RNA (snRNA) was probed as a loading control.
Microarray analysis

Three biological replicates of wild-type (Col) plants and two biological replicates of *polV* and *rdd* mutants (3-week-old plants grown on MS agar plates) were used in this analysis. Funding constraints enabled us to only include two biological replicates for the *polV* and *rdd* mutant plants. Total RNA was extracted from the whole plant tissues (10-12 plants in each replicate sample) using the RNeasy plant mini kit (Qiagen, Hilden, Germany) which includes a DNaseI treatment step. The yields of total RNA were measured using a NanoDrop (Thermo) at a wavelength of 260 nm and the quality of the samples was assessed by measuring the A260/A280 ratio (a ratio > 1.8 was deemed acceptable).

Microarray analysis was performed by Nimbelgen (Iceland). The total RNA was labeled and hybridized onto an *A. thaliana* 12x135K microarray, followed by washing and scanning. The resulting expression values were normalized by quantile normalization and the Robust Multichip Average (RMA) algorithm (Nimblegen) (Irizarry et al., 2003).

Two-way ANOVA was used to identify the differentially expressed genes between the wild-type, *polV* and *rdd* mutant plants. A *P*-value of 0.05 was used as a cut-off value, and a multiple testing correction using the Benjamini and Hochberg false discovery rate was applied to the data (Benjamini and Hochberg, 2005).

The likely biological functions of the differentially expressed genes were determined using the gene ontology (GO) program (http://www.arabidopsis.org/tools/bulk/go/index.jsp) available on the Arabidopsis Information Resource website.
Detection of AGO4 protein expression in Arabidopsis by Western blotting

Anti-FLAG tag antibodies were obtained commercially from Sigma-Aldrich. Protein extracts were prepared from whole plant tissues, as described by Li et al. (2006) using protein extraction buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.1% NP-40) containing fresh DTT (2 mM), PMSF (1 mM), pepstatin (0.7 mg/ml), MG132 (10 mg/ml), and protease inhibitor cocktail (Roche); and then centrifuged twice (13,000 rpm at 4°C).

The protein concentrations were determined using the BioRad Protein Assay. Equal amounts of protein were loaded for each sample and the proteins were resolved on 8% SDS polyacrylamide gels, transferred to Immobilon P membranes (Millipore), incubated with monoclonal anti-FLAG M2 antibody (1:1000; F1804, Sigma-Aldrich) followed by anti-mouse Ig HRP conjugate (1:5000; Chemicon, Victoria, Australia), and the bands were detected by chemiluminescence.

2.11 General molecular cloning techniques

2.11.1 Ligation of PCR fragments into the pGEM®-T Easy vector

The vector pGEM®-T Easy (Promega) was used as a cloning vector to facilitate sequencing and probe synthesis. The PCR products of PR1, AGO4, WRKY70 and WRKY33 (4.0 μl each) were mixed with 0.5 μl of the supplied vector, 5.0 μl of 2x ligation buffer and 0.5 μl of T4 DNA ligase in a total volume of 10 μl, incubated for at least 2 h or overnight at RT, then transformed into E. coli.
2.11.2 Transformation of *E. coli* DH5α electro-competent cells

One microlitre of ligation product was used to transform 50 μl of *E. coli* DH5α electro-competent cells by electroporation at 2.0 kV (Electro Cell Manipulator, ECM-395, BTX), then 450 μl chilled LB was added immediately, the mixture was transferred to a 1.5 ml microfuge tube and shaken at 200 rpm for one hour at 37°C. The bacterial suspension was plated onto LB plates containing 50 μg/ml (LB-Amp) containing 20 μl X-gal (40 mg/ml) and 10 μL 20% (IPTG) and incubated at 37°C overnight for blue/white selection based on the lac-Z operon of the pGEM-T Easy vector.

2.11.3 Screening for positive colonies

White colonies and a single blue insert-negative control colony were screened for the presence of the inserted DNA fragments using PCR. The PCR products were electrophoresed on an EtBr-stained 1.0% agarose gel in 1x Tris/Borate/EDTA (TBE) and visualised using a UV transilluminator. The positive colonies were used to inoculate 5.0 ml LB-Amp (50 μg/ml) cultures, and cultured at 37°C overnight (approximately 16 h) with shaking at 200 rpm.

2.11.4 Extraction of plasmid DNA

Plasmid DNA was extracted from the overnight cultures using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. The plasmid DNA preparations were resuspended in 50 μl of the provided Elution Buffer (Qiagen) or TE buffer containing 20 μg/ml RNase A.
2.11.5 Restriction enzyme digestion to confirm the presence and orientation of the inserted DNA fragments in pGEM®-T Easy

To confirm the presence of an insert, pGEM®-T Easy was digested using EcoR I. To determine the orientation of the inserts within pGEM®-T Easy, which was necessary for probe construction, an insert-specific restriction enzyme digest was designed using VectorNTI AdvanceTM 11 (Invitrogen) and performed using New England Biolabs and Fermentas restriction enzymes and buffers. When double or triple digests were required, the buffer which maintained the highest activity for all of the restriction enzymes was chosen, and the double or triple digest was carried out in a single incubation. All digests were carried out at 37°C, the optimal temperature for the enzymes used.

2.11.6 Sequencing of positive colonies

The plasmid DNA from the positive colonies was sequenced to ensure that the inserted DNA fragments had the correct sequence. A standard cycle sequencing procedure using a T7-specific primer for pGEM®-T Easy vector was used. The reaction products were purified by the addition of 1.5 μl NaOAC and 20 μl of 100% ethanol. After mixing, the mixture was incubated in the dark for 20 min at RT, centrifuged at 16,000 g for 15 min at RT, all traces of the supernatant were removed using a pipette, 250 μl ice-cold 70% ethanol was added and the was tube centrifuged at 4000g for 10 min to wash the DNA pellet. All traces of ethanol were removed by pipetting and the pellet was air-dried for 10 min at RT. The purified sequencing reactions were analysed by the Biomolecular Resource Facility at the John Curtin School of Medical Research,
ANU. The sequences of PR1 and AGO4 were confirmed using the alignment module of VectorNTI AdvanceTM 11 (Invitrogen).
CHAPTER 3: Identification of RNA silencing factors with a potential role in plant defence against pathogens in *Arabidopsis thaliana*
3.1 Introduction

A major function of the RNA silencing mechanisms in plants is to defend the host genome against invading nucleic acids. Invaders such as transgenes, viruses and transposons can be silenced via RNA silencing mechanisms (Wassenegger et al., 1994; English et al., 1996; Wassenegger, 2005; Eamens et al., 2008). As discussed in the literature review, recent studies have provided evidence that both transcriptional gene silencing (RdDM and chromatin remodelling) and posttranscriptional gene silencing (miRNA and siRNA) pathways play a role in plant defence against non-viral pathogens. The mechanisms of RNA silencing-mediated defence against viral pathogens are well understood; however, it remains largely unknown how RNA silencing is involved in plant defence against non-viral pathogens.

In this project, *Arabidopsis-F. oxysporum* was used as a host-pathosystem to investigate the role of RNA silencing in plant defence against non-viral pathogens. This chapter describes the screening of *Arabidopsis* RNA silencing mutants for resistance/susceptibility to *F. oxysporum* infection. Additionally, some preliminary results from a screen of *Arabidopsis* RNA silencing mutants infected with *A. tumefaciens* are described. In particular, the study focuses on the effect of loss-of-function mutations in the *de novo* DNA methylation (*PolV*) and demethylation (*RDD, DML2* and *DML3*) pathways on the plant response to *F. oxysporum*.

In this chapter I describe work that has:

1. Identified RNA-directed DNA methylation and demethylation-associated factors in *Arabidopsis* which are potentially involved in antifungal resistance to *F. oxysporum*. 


(2) Quantified fungal biomass in wild type and mutant plants with altered phenotypic responses to *F. oxysporum*.

(3) Performed grafting to investigate the role of roots and shoots in the mediation of plant defence against *F. oxysporum*.

(4) Investigated the possible role of RNA silencing factors in the mechanism of disease resistance to *A. tumefaciens*. 
3.2 Results

3.2.1 Mutations in RdDM pathway factors alter the plant response to *F. oxysporum* infection

In order to dissect the role of *de novo* methylation pathway factors in plant defence against non-viral pathogens, we assayed the *Arabidopsis* *de novo* methylation pathway mutant *polV* for altered resistance or susceptibility to *F. oxysporum*. PolV is a downstream component of the RdDM pathway and interacts with AGO4 to guide the *de novo* methylation of DNA by DRM2.

In the first series of experiments, 4 week-old wild-type (wt) and *polV* mutant plants were grown in soil and then inoculated with *F. oxysporum* or mock treated with water as a control, and the disease symptoms were evaluated at 7 days post inoculation (dpi). Mock-treated *polV* plants did not display any visible developmental defects or abnormalities and looked similar to mock-treated wild-type plants (Fig. 3.1a). At 7 dpi, *polV* mutant plants displayed a severely diseased phenotype with extensive yellowing and wilting of the leaves, in contrast to wild-type plants where a mild yellowing and wilting phenotype was observed in only a small number of plants (Fig. 3.1b).

A sugar-free MS agar based assay system was developed by Tuan Le in our laboratory to screen *Arabidopsis* for *F. oxysporum*-resistant or susceptible phenotypes. Four week-old wild-type and *polV* mutant plants were infected with *F. oxysporum* on sugar-free MS plates. Consistent with the results of the soil infection experiment, the *polV* mutant plants displayed stronger disease symptoms than wild-type plants at 7 dpi (Fig. 3.1c).
Infection of younger plants (2 weeks old) with a lower dose of *F. oxysporum* revealed a similar phenotypic difference between *polV* and wild-type. At 6 dpi, the *polV* mutant plants started to display slight vein clearing and yellowing of the leaves, while no symptoms were apparent on the leaves of wild-type plants (Fig. 3.2a). At 9 dpi, the *polV* mutants showed severe disease progression, with most of the leaves showing yellowing and vein clearing (Fig. 3.2b). In contrast, wild-type plants at the same stage showed only moderate leaf yellowing, and were subsequently able to grow and set seeds. The increased susceptibility of *polV* to *F. oxysporum*, in comparison to wild-type plants, suggests that the *de novo* methylation factor Pol V is critical for disease resistance against *F. oxysporum* in *Arabidopsis*. Therefore, it is possible that components of the RdDM pathway are involved in plant antifungal defence.

### 3.2.2 The *Arabidopsis* triple mutant, defective in the DNA demethylation factors ROS1, DML2 and DML3, is highly susceptible to *F. oxysporum* infection

In addition to the RdDM pathway factor PolV, I investigated the potential role of the demethylation pathway in plant defence against *F. oxysporum*. REPRESSOR OF SILENCING (ROS1), together with DEMETER-LIKE (DML) enzymes, function in DNA demethylation (Penterman et al., 2007). A *ros1/dml2/dml3* triple mutant, kindly provided by Jian-Kang Zhu, University of California, USA, was used for this study. For convenience, the triple mutant will be referred to as *rdd* from this point onwards.

When grown and infected in soil, mock-treated *rdd* plants did not show any visible abnormalities or phenotype in comparison to wild-type plants (Fig. 3.1a). However, *rdd* exhibited a strong
susceptibility to *F. oxysporum* infection, with severe yellowing of the leaves observed at 7 dpi (Fig. 3.1b).

*F. oxysporum* infections were then performed on sugar-free MS plates. The different response of *rdd* and wild-type plants to *F. oxysporum* infection was more evident when assayed on MS plates than in soil (Fig. 3.1c). On MS agar plates, the *rdd* plants were hyper-susceptible to *F. oxysporum*, with most plants showing severe yellowing of the leaves at 7 dpi (Fig. 3.1c), in contrast to wild-type plants which showed little yellowing at this stage. At 10 dpi, the *rdd* mutant plants showed severe leaf yellowing, or even death with obvious fungal growth, while the wild-type plants exhibited few symptoms of disease.

When younger plants (2-weeks old) were inoculated with a lower dose of *F. oxysporum*, a similar phenotypic difference was observed between *rdd* mutant and wild-type plants. The *rdd* mutants displayed severe necrosis and many of the infected plants died (Fig. 3.2b). In contrast, wild-type plants showed only a moderate level of leaf yellowing, and eventually grew to maturity and set seed. These results suggested that components of the demethylation pathway are involved in the plant response to *F. oxysporum* infection.
Figure 3.1 *Arabidopsis* de novo methylation (*polV*) and demethylation (*rdd*) mutants display increased susceptibility to *F. oxysporum*. Four week old wild-type, *polV* and *rdd* plants were inoculated with *F. oxysporum* (10^7 spores/ml) and the disease severity was assessed 7 days post inoculation. a. Mock treated plants; b. *F. oxysporum* infected plants in soil; c. *F. oxysporum* infected plants on MS agar.
Figure 3.2 *Arabidopsis de novo* methylation (*polV*) and demethylation (*rdd*) mutants display susceptible phenotypes to *F. oxysporum*. Two-week old wild-type, *polV* and *rdd* plants were inoculated with *F. oxysporum* (10^6 spores/ml) and the disease severity was assessed at 6 (a) and 9 (b) days after inoculation. dpi – days post inoculation.
3.2.3 Both the roots and shoots contribute to plant response to *F. oxysporum* infection in *rdd* and *polV* mutants

After identifying methylation and demethylation *Arabidopsis* mutants with altered responses to *F. oxysporum* infection, we investigated whether the susceptibility or resistance to this pathogen was conferred by the roots or shoots. *F. oxysporum* infects plants via the roots; therefore, it was important to determine whether the roots play a major role in the phenotypic response of *rdd* and *polV* mutants to *F. oxysporum*. *Arabidopsis* shoots and roots can be grafted; therefore, we used a grafting technique to study the role of roots and shoots in the plant response to *F. oxysporum* (Turnbull et al., 2002).

As shown in Fig. 3.3a and Fig. 3.3b, self-grafting (control grafts) did not affect the resistance or disease phenotype of wild-type, *polV* or *rdd*. At 14 dpi, the wild type control grafts (wild type scion grafted onto wild type rootstock) showed a moderately resistant phenotype; whereas the *polV* and *rdd* grafts (*polV* scions on *polV* root stock or *rdd* scions on *rdd* rootstock) displayed susceptible phenotypes.

Reciprocal grafts between wild-type and *rdd* or *polV* plants were then assayed. At 14 dpi, the grafts between wild-type scion and *rdd* root stock were more susceptible to *F. oxysporum* than the moderately resistant wild-type control grafts (Fig. 3.3a); however, grafts between wild-type scion and *rdd* root stock were less severely diseased than the *rdd* control grafts. Similarly, the reciprocal grafts between the *rdd* scion and the wild-type root stock were more susceptible to *F. oxysporum* than the moderately resistant wild-type control grafts, but less severely diseased than the *rdd* control grafts (Fig. 3.3a).
Similarly to *rdd* and wild-type grafts, grafts between wild-type scions and *polV* root stocks were more susceptible to *F. oxysporum* at 14 dpi than wild-type control grafts, but less severely diseased than *polV* control grafts (Fig. 3.3b). Additionally, grafts between *polV* scions and wild type root stocks were more susceptible to *F. oxysporum* than the moderately resistant wild-type control grafts (Fig. 3.3b), and less severely diseased than *polV* control grafts.

The intermediate phenotype of the reciprocal grafts, in comparison to the control grafts, suggests that the response to *F. oxysporum* infection is mediated by both the roots and shoots in *polV* and *rdd* mutants. Therefore, overall plant defence response to the *F. oxysporum* pathogen is likely to be controlled at the whole plant level, and not in a root or shoot-specific manner.
Figure 3.3a Control grafts and reciprocal grafts were made between wild-type and rdd plants. These grafts were inoculated with F. oxysporum and assessed at 14 days post-inoculation. Scions and rootstocks are indicated by the labels above and below the lines, respectively. In all grafting experiments 3 week-old grafts were infected.
Figure 3.3b Control grafts and reciprocal grafts were made between wild-type and polV plants. These grafts were inoculated with *F. oxysporum* and assessed at 14 days post-inoculation. Scions and rootstocks are indicated by the labels above and below the lines, respectively. In all grafting experiments 4 week-old grafts were infected.
3.2.4 The susceptible mutants *polV* and *rdd* accumulate less fungus in their shoot tissue compared to the wild-type plants but more in root tissue

Accurate detection and quantification of pathogen titer or biomass in infected plants is a critical step in the monitoring of disease resistance. Various nucleic acid-based techniques, such as PCR, can be employed to detect and quantify pathogen genomic DNA (Nicholson et al., 2003; Oliver et al., 2008). PCR offers both sensitive and specific quantification (Oliver et al., 2008). In this study, PCR was used to assess pathogen colonization in infected plants, by quantifying the presence of pathogen DNA using *F. oxysporum*-specific gene primers.

To determine whether accelerated post-penetration fungal growth was responsible for the observed susceptibility of *polV* and *rdd* to *F. oxysporum*, the fungal biomass was measured in DNA extracted from the shoot tissue of infected wild-type, *polV* and *rdd* mutants using quantitative real-time PCR (qPCR), using specific primers for the *F. oxysporum cutinase* and *GPD* genes (Thacher et al., 2009). These genes consist of highly conserved internal transcribed spacer regions (ITS) specific to *Fusarium oxysporum* (Gurjar et al., 2009). Sampling was conducted 7, 10 and 14 dpi (Fig. 3.4). At 7 dpi, *polV* and *rdd* mutants displayed more severe symptoms than the wild-type plants (Fig. 3.4a). At this stage, quantification of the *cutinase* gene indicated a slightly lower level of fungal accumulation in *polV* than wild-type plants; however, the *GPD* gene was amplified to a similar level in *polV* and wild-type plants (Fig. 3.5b). Fungal accumulation in the shoots of *rdd* was also slightly lower than wild-type plants using *F. oxysporum*-specific primers (Fig. 3.5a and Fig. 3.5b). At 10 dpi, when *polV* and *rdd* mutants displayed extensive chlorosis relative to wild type plants (Fig. 3.4b), a significantly lower fungal titer was observed in the shoots of both *polV* and *rdd* in comparison to wild-type plants (Fig.
3.5a and Fig. 3.5b). A similar pattern of fungal titer was observed at 14 dpi, when severe necrosis was observed in polV and rdd mutants (Fig. 3.4c). These results suggest an inverse correlation between the severity of disease and fungal accumulation in the shoots of *F. oxysporum*-infected *Arabidopsis*.

*F. oxysporum* is a root-infecting pathogen which causes necrosis in infected tissues; therefore, we investigated fungal accumulation at 10 dpi in the root of wild-type plants and susceptible mutants. At this stage, polV and rdd mutants showed severe leaf necrosis compared to wild type plants (Fig. 3.6b). polV and rdd accumulated higher levels of fungal biomass in the root tissue than wild-type plants (Fig. 3.6a); therefore, the level of fungal biomass in the roots correlated with the disease susceptibility of *Arabidopsis* lines to infection with *F. oxysporum*. 
Figure 3.4 Assessment of disease severity in wild-type, polV and rdd plants inoculated with *F. oxysporum* (10^6 spores/ml). a. 7 dpi, b. 10 dpi, c. 14 dpi. dpi - days post inoculation.
Figure 3.5 Estimation of *F. oxysporum* biomass in wild type, *polV* and *rdd* shoot tissue.

Relative abundance of fungal biomass by quantitative real-time PCR using *Fusarium*-specific primers for (a) Cutinase and (b) *GPD* in the shoots of inoculated plants at 7,10,14 days post infection (dpi). Relative abundance value set at 1 for wild type.
Figure 3.6 Estimation of \( F. \) oxysporum biomass in wild-type, \( polV \) and \( rdd \) root tissue. (a) Fungal biomass was quantified in wild-type, \( polV \) and \( rdd \) mutant plants by assaying \( F. \) oxysporum specific gene Cutinase in the roots of inoculated plants by using semi-quantitative RT-PCR 10 days following infection. The host \textit{Arabidopsis} housekeeping gene \( iASK \) was amplified as loading control. (b) Diseased phenotype 10 days following \( F. \) oxysporum infection.
3.2.5 Role of RNA silencing factors in plant defence against Agrobacterium tumefaciens

The soil pathogen Agrobacterium tumefaciens can transfer a small segment of DNA (known as T-DNA) from a resident Ti plasmid into the plant genome. T-DNA–encoded oncogenes promote plant cell proliferation by altering the auxin/cytokinin balance, such that bacteria thrive on the resulting tumor by metabolizing the nutrients, termed opines, which are produced by T-DNA–encoded enzymes (Gelvin, 2005). Non-oncogenic or 'disarmed' T-DNAs have been widely used for transient and stable plant transformation for several decades (Gelvin, 2005); however, transgenes are often poorly expressed, or not expressed at all, due to RNA silencing (Dunoyer et al., 2006).

The activities of four distinct Dicer-like (DCL) proteins define various endogenous silencing pathways in Arabidopsis (Xie et al., 2004). DCL1 is necessary for the accumulation of miRNAs, and plant growth and development (Yu et al., 2005). DCL2 produces 22-nt siRNAs which may mediate antiviral defence. DCL3, together with RDR2, produces 24-nt siRNAs that guide epigenetic modifications, particularly at transposon loci and DNA repeats, to result in transcriptional gene silencing (Gasciolli et al., 2005). Finally, DCL4 and RDR6 function in the synthesis of 21-nt trans-acting siRNAs, which mediate juvenile-to-adult phase transitions (Xie et al., 2004).

Unlike endogenous silencing pathways, the plant factors required for RNA silencing triggered by exogenous T-DNA–based constructs are yet to be fully characterised. For instance, RDR6 is required for sense-transgene silencing; however, it is not known which DCL(s) are involved in downstream signalling (Dalmay et al., 2000). Additionally, the Turnip crinkle virus P38 protein
specifically inhibits the production of siRNAs from inverted-repeat or sense transgenes; however, P38 has little or no effect on endogenous RNA silencing pathways in Arabidopsis (Dunoyer et al., 2004).

In this study we screened the siRNA biogenesis mutants dcl2/4, dcl4 and dcl2, the de novo methylation pathway mutants pollIV and rdr2, and the triple demethylation pathway mutant rdd for altered response to the virulent A. tumefaciens. This virulent Agrobacterium strain carries a wild-type T-DNA encoding tumor-inducing genes. Formation of tumors on A. tumefaciens-inoculated Arabidopsis roots was used to monitor the efficiency of Agrobacterium infection and Agrobacterium-mediated transformation.

A MS agar-based assay system was used to screen Arabidopsis mutants deficient in siRNA biogenesis or the RdDM pathway for altered response to A. tumefaciens. Around 40-50 root segments from two week-old wild-type, various DCL mutants, pollIV or rdr2 mutants were infected with A.tumefaciens and transferred to MS plates for tumor formation and growth.

DCL mutants (dcl2, dcl4 and dcl2/4) formed more tumors than the wild-type (Fig 3.7a), with dcl2 and dcl2/4 mutants forming the highest number of tumours. More strikingly, the tumors derived from the dcl4 mutants, especially the dcl2/4 mutants, were significantly larger than the tumors formed by wild-type plants (Fig. 3.7b). The size of the tumors formed by the wild-type were highly variable, while tumors formed by the dcl4 and dcl2/4 mutants were uniformly larger and greener, and most of these tumors eventually developed shoots (Fig. 3.7b). It is likely that the optimal balance of cytokinin and auxin may have led to the development of shoots and
organogenesis in dcl mutants relative to the wild-type plants. This result indicates that these siRNA pathway mutants were more susceptible to A. tumefaciens infection and A.tumefaciens-mediated transformation. It also suggests that the T-DNA-encoded tumor-inducing genes are less silenced in the dcl mutants, resulting in more frequent and rapid growth of tumors.

Another important observation from the A. tumefaciens infection experiment was that the RdDM pathway mutants nrpd1a (polIV), nrpd2a (polIV and polV) and rdr2 consistently formed tumors more frequently (on a quantitative basis) than the wild-type control (Fig. 3.7c), suggesting a higher frequency of T-DNA integration. The number of tumors per unit length of root segment in the RdDM mutants was greater than the dcl mutants. However, unlike the dcl mutants which formed large tumors, the RdDM pathway mutants, especially the upstream factor mutants polIV and rdr2, formed small (on a qualitative basis), uniform slow-growing tumors compared to the wild-type (Fig. 3.7d). This result suggests that the RdDM pathway affects not only A.tumefaciens infection and T-DNA integration, but also the development of tumor cells. Taken together, our results indicate that both the siRNA and the RdDM pathways influence the interaction between A. tumefaciens and Arabidopsis.
Figure 3.7a Frequency of tumor formation on roots of wild-type and siRNA pathway mutants dcl4, dcl2/4 and dcl2 at 14 days following *A. tumefaciens* infection.
Figure 3.7b The size of tumor formation on roots of wild-type and siRNA pathway mutants dcl4, dcl2/4 and dcl2 at 28 days following *A. tumefaciens* infection.
Figure 3.7c The frequency of tumor formation on roots of wild-type and RdDM pathway mutants nrpd2a (polIV and polV), nrpd1a (polIV) and rdr2 at 14 days following A. tumefaciens infection.
Figure 3.7d The size of tumor formation on roots of wild-type and RdDM pathway mutants nrpd2a (polIV and polV), nrpd1a (polIV) and rdr2 at 28 days following *A. tumefaciens* infection.
3.3 Discussion

Small non-coding RNAs regulate a multitude of biological processes in plants, including sustaining the integrity of the genome, and regulating development, metabolism and the response to altered environmental conditions. Increasing evidence indicates that small non-coding endogenous plant RNAs, including miRNAs and siRNAs, are integral components of the plant defence against microbial pathogens.

This study aimed to identify factors associated with small RNAs which may be involved in plant immunity against non-viral pathogens using a genetic screen. We identified that RNA polymerase V, a downstream component of the RdDM pathway, and the DNA demethylation enzymes are critical for plant defence against *F. oxysporum*.

Our observations are consistent with a recent finding which demonstrated a role of PolV in the plant defence against non-viral pathogens. *polV* mutant plants have an enhanced susceptibility to the necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*, whereas *polV* plants display enhanced resistance against the bacterial pathogen *Pseudomonas syringae* (López et al., 2011). The PolV-AGO4 protein complex facilitates *de novo* methylation as part of the RdDM pathway (Daxinger et al., 2009). Interestingly, *ago4* mutant plants have severely compromised resistance to the bacterial pathogen *Pseudomonas syringae* (Agorio and Vera, 2007). Thus, the PolV and AGO4 proteins offer contrasting defence responses to *Pseudomonas syringae*. These observations suggest that the protein complex comprised of proteins such as PolV and AGO4 which act downstream of the RdDM pathway plays a specific role in plant
defence against non-viral pathogens. Overall, our results demonstrate the importance of the methylation factor PolV and the demethylases in plant defence against F.oxysporum.

Resistance to microbial infections requires the transcription of a wide range of genes encoding regulatory and antimicrobial proteins. Plant responses to the environment and many physiological processes can alter DNA remodelling; therefore, altered transcriptional control during the plant response to infection is likely to result from a change in chromatin state and DNA modifications (Kouzarides, 2007). Changes in higher-order chromatin structure, such as chromatin condensation, occur during plant cell death induced by fungal toxins (Navarre and Wolpert, 1999; Liang et al., 2003). Various chromatin remodelling-associated genes are also involved in plant defence against bacterial and fungal pathogens in Arabidopsis (Alvarez-Venegas et al., 2006; Durrant et al., 2007). For instance, histone methyltransferase SDG8 is involved in the establishment of a chromatin state that is required for inducible defence against the necrotrophic fungal pathogens in Arabidopsis (Berr et al., 2010). Histone deacetylase 19 (HDA19) enhances plant resistance to Alternaria brassicicola (Zhou et al., 2005); whereas knockdown of HDA6 impairs the basal expression of defence-related genes (Wu et al., 2008). Our observations further underline the importance of epigenetic factors in the regulation of plant defense against F. oxysporum.

To further understand the role of methylation and demethylation pathways in the development of resistance to fungal infections, a grafting study was performed to define the role of roots and shoots in the observed susceptibility of polV and rdd mutants to F.oxysporum. Plants have a variety of defence responses, which can occur above ground (AG) in the leaves and below
ground (BG) in the roots (Van Dam and Bezemer, 2006). BG pathogen infection can induce AG defence responses, and vice versa. As *F. oxysporum* is a root-infecting pathogen which invades the shoot at the late stages of infection, resistance to this pathogen may be determined by the root, shoot, or both the root and shoot. Our grafting experiment shows that reciprocal grafts between wild-type and susceptible mutants (*polV* or *rdd*) displayed an intermediate phenotype in response to *F. oxysporum* infection, suggesting that resistance to *F. oxysporum* occurs at the whole plant level and is mediated by both the roots and shoots in *Arabidopsis*. The roots and shoots constantly communicate with each other about their current status, via hormones, and optimal incorporation of this information is critical for maximizing plant fitness in unpredictable AG and BG environments (Van Dam and Bezemer, 2006). Thus, the intermediate phenotype observed in these reciprocal grafts may indicate that the roots or shoots of the moderately resistant wild-type plant in the reciprocal grafts impart resistance to the whole grafted plant through hormonal signals.

Furthermore, in this study, a nucleic acid-based technique (PCR) was used to quantify fungal biomass in the shoots and roots of infected plants following *F. oxysporum* infection. At 10 dpi, the susceptible *polV* and *rdd* mutant plants accumulated more fungus in their roots than did the more resistant wild-type plants.

The rapid development of cell death at or around sites of infection is a common feature of disease resistance, and results in necrosis of the tissue; a phenomenon known as the hypersensitive response (HR) (Morel and Dangl, 1997). The HR functions to restrict the growth and spread of pathogens (Morel and Dangl, 1997), and is considered to be one of the most
important factors which impedes the growth of biotrophic pathogens (Greenberg, 1997). However, HR has also been observed when hemi-biotrophs such as *F. oxysporum*, or necrotrophs like *Botrytis cinerea* and *Sclerotinia sclerotiorum* interact with *Arabidopsis* (Govrin and Levine, 2000). Previous findings show that these necrotrophs utilize the plant HR to induce rapid colonization to complete their lifecycle (Govrin and Levine, 2000). Thus, it is plausible that *F. oxysporum* may have elicited a severe HR response in the susceptible mutants relative to the wild-type plants following infection (Johal et al., 1995), leading to necrosis and higher fungal biomass accumulation in the roots of susceptible plants. This tissue necrosis followed by the HR response may have provided an ideal or conducive environment for *F. oxysporum* to complete its lifecycle on dead tissue.

In addition, PCR analysis also showed that *F. oxysporum* accumulation was reduced in the shoots of the *polV* and *rdd* mutants in comparison to the wild-type plants, which is in contrast to the observation in the root tissue. One possible explanation is that the higher level of fungal accumulation in the roots of *polV* and *rdd* mutants led to severe clogging of the vasculature, thereby affecting the movement of fungal hyphae and the transport of nutrients and water from the roots to shoots, resulting in the wilting of the plant.

As an initial effort to investigate the role of RNA silencing in the plant defence against non–viral pathogens, several *Arabidopsis* RNA silencing mutants were screened using *A. tumefaciens* infections. The siRNA pathway mutants *dcl2*, *dcl4* and *dcl2/4* were more susceptible to *A. tumefaciens* infection and *A. tumefaciens*-mediated transformation than wild-type control plants. More significantly, *dcl* mutants developed a larger number of tumors, and larger tumors
than wild-type plants, suggesting that the tumor-inducing genes derived from the Ti plasmid of
A.tumefaciens were silenced to a lesser extent in dcl mutants, due to loss of function in DCLs,
which results in increased cytokinin production and more rapid tumor growth. Increased tumor
formation in dcl mutants is consistent with a previous report, which indicated that the
A.tumefaciens-mediated transformation efficiency increases in plants expressing the silencing
suppressor protein p38 (Dunoyer et al., 2006).

Infection of the RdDM pathway mutants provided interesting results. These mutants, especially
the upstream 24nt siRNA biogenesis mutants polIV and rdr2, displayed a very high frequency of
tumor formation in comparison to the wild-type control, indicating a higher level of T-DNA
integration into the genome of these mutant lines. It is possible that loss of function of the RdDM
pathway factors alters or loosens (opens) the chromatin structure, which may be more conducive
to T-DNA integration. Another possibility is that integrated T-DNA genes are less likely to be
silenced via transcriptional inactivation due to the loss of RdDM factors, allowing increased
tumor formation in transformed cells. Previous studies have suggested that transgenes which
integrate into hetrochromatic regions of plant genomes tend to be transcriptionally silenced
(Schubert et al., 2004); a phenomenon known as the position effect. It is possible that transgenes
integrated into the hetrochromatin regions of RdDM mutant genomes remain active and are
expressed, which in the case of wild-type T-DNA allows the expression of tumor-inducing genes
leading to frequent tumor formation.

In conclusion, investigation of the Arabidopsis-F.oxysporum and Arabidopsis-A. tumefaciens
systems indicated that methylation and demethylation factors are important components of the
plant defence against non-viral pathogens. The *Arabidopsis*-*A. tumefaciens* pathosystem is worth pursuing in the future, as it offers a promising system to study plant-pathogen interactions and has potential implications for improving the efficiency of *A. tumefaciens*-mediated plant transformation. However, due to time constraints, the remainder of the thesis mainly focuses on the *Arabidopsis*-*F. oxysporum* interaction.
CHAPTER 4: Analysis of genome-wide transcriptome changes of

*Arabidopsis polV* and *rdd* mutants
4.1 Introduction

Through the process of natural selection, plants have developed sophisticated defense mechanisms in order to protect themselves, whereas plant pathogens and invaders of all types have evolved in ways that allow them to overcome those defenses. The evolutionary arms race has resulted in a large variety of constitutive plant defense mechanisms such as physical and chemical barriers, as well as inducible plant defense responses that become activated only upon attack (Dicke and Hilker, 2003).

The cell wall itself is the first line of defense, as it provides cells a physical barrier against attack from pathogens and microorganisms. It also offers protection against mechanical stress to the cell (Collinge, 2009). For instance, following pathogen attack, plants often deposit callose-rich cell wall appositions at sites of infection or pathogen penetration, accumulate secondary metabolites and synthesize lignin-like polymers to strengthen the wall (Hématy et al., 2009). Pathogen attack triggers complex signaling cascades regulated by signaling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). The result of signaling cascade activation is both the expression of defense-related genes such as those encoding pathogenesis-related (PR) proteins, and the production of antimicrobial secondary metabolites. Resistance against certain groups of pathogens depends on the selective activation of particular defence circuits in plants (Jones and Dangl, 2006; Bent and Mackey, 2007). The formation of lignin as a defense mechanism against fungal pathogens has long been established (Vance et al., 1980). There is a good correlation between the rapid induction of secondary metabolites such as lignin and resistance to fungi (Habereder et al., 1989; Southerton and Deverall, 1990). Other secondary metabolites like terpenes have also been shown to be important factors in resistance to several
pathogens, especially those secreted by insects (Harborne, 1988). The insecticidal activity of the terpenes is specific to the terpene the plant generates: The effects can either be due to the action of terpenes as toxins, anti-feedants, or deterents, or as modifiers of insect development, e.g. sterols such as the phytoecdysones (Harborne, 1988). In desert plants, a number of terpenoids have been found to be good insect deterents (Rodriguez and Hedin, 1983). Similarly, in cotton that was resistant to infection by *Verticillium dahliae* there was a strong induction of terpenoids which were positively correlated with resistance to the fungus (Garas and Waiss, 1986). Plants produce primary and secondary metabolites which encompass a wide array of functions (Zwenger and Basu, 2008). Genes involved in secondary metabolite production include those encoding enzymes such as terpene synthases, which generate terpenes in response to stress, such as the case when acting as a deterrent against insects and herbivores (Keeling and Bohlmann, 2006). Volatile and non-volatile terpenes are implicated in the attraction of herbivores of both pollinators and predators, in protection against photooxidative stress, in mediating thermotolerance, and in direct defense against microbes and insects.

Mounting evidence suggest that lipid derivatives produced by host plants are crucial signals that modulate host-pathogen communications (Burow et al., 1997; Calvo et al., 1999; Tsitsigiannis and Keller, 2006). It is proposed that lipid-mediated signaling in the host governs the outcome of host-pathogen interaction, resulting in activation of plant defense response genes and/or promoting fungal vegetative growth, sporulation, and mycotoxin production (Gao and Kolomiets, 2009).
Recently, non-coding small RNAs that are responsible for directing chromatin modifications have also been implicated in triggering the defense response against pathogens, however defense regulation mediated by endogenous small RNAs have been implicated in only few cases (Alvarez-Venegas et al., 2006; Walley et al., 2008). Studies have begun to look at how chromatin structure, and modification thereof, affects the expression of defence genes. The structure of chromatin influences several genomic functions: At a basic level, 147bp of DNA are wrapped around core histone proteins forming a functional chromatin unit, a nucleosome. Nucleosomes are separated by unwrapped DNA approximately 10-50bp in length that are associated with linker histone H1. This packaging of DNA can prevent access of the transcription machinery to DNA. The chromatin needs to be relaxed in order to make this unpackaged DNA available to the transcription machinery. Chromatin unfolding involves modification of histone proteins or DNA methylation changes and requires the action of an ATP-dependent remodelling complex (de la Serna et al., 2006). Many of these epigenetic modifications are now linked to plant defence against pathogens through turning inducible defense genes on or off via structural changes in chromatin (Walley et al., 2008).

In the previous chapter, evidence was presented indicating that PolV (methylation) and RDD (demethylation) factors are required for plant defence against \textit{F. oxysporum}, suggesting that some plant defence-related genes are regulated by DNA methylation and demethylation mechanisms. In this chapter, microarray analysis was used to survey genome-wide transcriptional changes in the \textit{polV} and \textit{rdd} mutants, with the objective of identifying defence-related genes that might be regulated by PolV and RDD. The resulting data set provides valuable
information on how de novo DNA methylation and demethylation might play a role in plant disease resistance.

4.2 Results

The transcriptome of the Arabidopsis polV and rdd (ros1/dml2/dml3) mutants was analysed using microarray and compared with that of wild-type plants. Two biological replicates were analysed for each of the two mutants, and three replicates were analysed for the wild-type control. The result showed that 480 genes were upregulated and 752 genes were downregulated by ≥2 fold in the polV mutant compared to the wild-type (p < 0.05) (A list of highly altered genes is provided in Appendix IV). For the demethylation pathway mutant rdd, 70 genes were upregulated while 304 genes were downregulated compared to the wild-type (p < 0.05) (Appendix V). Thus, more genes were downregulated than upregulated in both polV and rdd mutants. In polV, a large proportion of genes that were upregulated are transposons (Appendix IV), consistent with the role of PolV in the transcriptional gene silencing of transposons and repetitive DNAs.

As polV and rdd mutants are susceptible to F. oxysporum, I focused our analysis on biotic stress-related genes that are altered in polV and rdd mutants and may contribute to the susceptibility of these mutants to infection. To gain functional insight from the transcriptional changes, we used the MapMan visualization tool (Usadel et al., 2005). This software package allowed us to categorize the up- or downregulated Arabidopsis genes into functional categories, that represent a particular cellular process, biological response or enzyme family. The genomic data sets can be displayed on pictorial diagrams representing different biological functions. By visualizing transcriptional changes, it becomes possible to discover patterns that are not immediately
obvious by studying individual genes. We used these diagrams to analyze the nature of the global
gene expression changes in the susceptible mutants \textit{polV} and \textit{rdd} as compared to the wild-type.
We found that the majority of the differentially expressed genes with a potential role in biotic
stress were downregulated in both \textit{polV} and \textit{rdd} mutants (Figs 4.1, 4.2). Of the differentially
expressed genes in \textit{polV}, all of those associated with salicylic acid (SA) and abscisic acid (ABA)
hormone signaling were down-regulated. In addition, a large percentage of the genes related to
ethylene (ET) hormone signaling (90%), cell wall synthesis (84%), auxin hormone signaling
(70%), other signaling genes (81%), transcription factors (86%), and secondary metabolism
(79%) were also downregulated(Fig 4.1). Among the differentially expressed genes in the \textit{rdd},
all the auxin signaling and secondary metabolism- related genes, as well as a large proportion of
genes related to cell wall synthesis (86%), signaling (87%), transcription factors (88%) and PR
proteins (71%) were downregulated (Fig 4.2).
Figure 4.1 Differential expression of biotic stress-related genes in the *polV* mutant: Changes in gene expression in *polV* mutant relative to wild-type. The plant's reaction to biotic stress involves: The initial signal input from the pathogen which is recognized by the related receptors (putative R genes) and triggers defence gene regulation and transcription of the cascade of the plant defence mechanism, including oxidative stress changes. Inside the cell, signals are transmitted that lead to the production of defence molecules (PR-proteins, heat shock proteins and secondary metabolites). Red blocks: Genes downregulated relative to wild-type. Blue blocks: Genes upregulated relative to wild-type.
Figure 4.2 Differential expression of biotic stress-related genes in the *rdd* mutant: Changes in gene expression in *rdd* mutant relative to wild-type. The plant's reaction to biotic stress involves: The initial signal input from the pathogen which is recognized by the related receptors (putative R genes) and triggers defence gene regulation and transcription of the cascade of the plant defence mechanism, including oxidative stress changes. Inside the cell, signals are transmitted that lead to the production of defence molecules (PR-proteins, heat shock proteins and secondary metabolites). *Red blocks*: Genes downregulated relative to wild-type. *Blue blocks*: Genes upregulated relative to wild-type.
Next, we analyzed the downregulated genes that were unique for polV and rdd mutants. In the polV mutant, 617 genes were uniquely downregulated, of these, 119 genes are biotic stress-related genes mainly associated with hormone signaling (15), cell wall synthesis (25), protein degradation (17) and signaling-related processes (34) (Fig 4.3).

In rdd mutant, 169 genes were uniquely down-regulated, of these, 38 genes (Fig 4.4) are biotic stress-related and are mainly associated with signaling (9), stress (6) and hormone related processes (5).

Since both polV and rdd mutants show increased susceptibility to F. oxysporum, we investigated genes that were commonly altered in both mutants. Using the MapMan tool, we identified a total of 135 genes that were commonly downregulated in polV and rdd (Fig 4.5). Of these, 45 genes are involved in biotic stress with many of these genes belonging to processes such as cell wall synthesis (21), stress (7), lipid metabolism (6), signaling (5) and secondary metabolism (4) and hormone related processes (2) (Fig 4.6).

The Mapman analysis identified 135 genes commonly downregulated in the polV and rdd mutants. Many of these genes belong to the processes such as lipid metabolism, cell wall synthesis, PR proteins that have some role in biotic stress response. A brief outline of some of the commonly downregulated biotic stress-related categories is listed below (section 4.2.1 to 4.2.5).
Figure 4.3 Biotic stress related genes that are uniquely downregulated in *polV* mutant relative to wild-type. MapMan was used to group the downregulated genes into functional classes. The pie chart shown here shows the number of genes in each class that were uniquely downregulated in the *polV* mutant compared to the wild-type.
Figure 4.4 Biotic stress related genes that are uniquely downregulated genes in $rdd$ mutant relative to wild-type. The pie chart shows the number of genes in each class that were uniquely downregulated in the $rdd$ mutant compared to the wild-type.
Figure 4.5 Venn diagram showing uniquely and commonly downregulated genes in *polV* and *rdd*. Number in bracket represents biotic stress related genes among uniquely and commonly downregulated genes.
**Figure 4.6 Biotic stress genes that are downregulated in both polV and rdd mutants**

MapMan was used to group the downregulated genes into functional classes. The pie chart here shows the number of genes in each class that were downregulated in both the polV mutant and the rdd mutant compared to the wild-type.
4.2.1 Fatty acid and lipid metabolism

Fatty acids are substrates for the biosynthesis of oxidized lipids. Moreover, fatty acids regulate the activity of enzymes involved in the generation of signaling molecules used in plant defense. Our microarray data revealed various genes in this functional category of lipid metabolism that were commonly downregulated in the *F. oxysporum*-susceptible mutants *polV* and *rdd* (Table 4.1). In addition, 2 lipid transfer proteins, namely LTP2 (at2g38530) and LTP4 (at5g59310), were significantly and uniquely downregulated in the *polV* mutant, whereas these 2 genes were downregulated less than 2 fold in the *rdd* mutant relative to that in the wild type. LTP2 and LTP4 belong to the PR14 class of PR proteins, which are known to possess antibiotic properties against the bacterial wilt pathogen *Ralstonia solanacearum*, as well as against *Fusarium solani* and *Plectosphaerella cucumerina* (Segura et al., 1993; Hernandez-Blanco et al., 2007). This significant downregulation of lipid-associated genes is indicative of the potential importance of PolV and RDD-associated pathways in the regulation of lipid metabolism. Further, it suggests the potential involvement of lipid metabolism in *Fusarium* resistance.

**Table 4.1 Commonly downregulated genes involved in lipid metabolism in rdd and polV**

<table>
<thead>
<tr>
<th>TAIR ID</th>
<th><em>rdd</em> (fold change)</th>
<th><em>polV</em> (fold change)</th>
<th>Related to</th>
</tr>
</thead>
<tbody>
<tr>
<td>at5g58050</td>
<td>2.3</td>
<td>3.43</td>
<td>Glycerol metabolic process, phosphorylation</td>
</tr>
<tr>
<td>at1g66120</td>
<td>5.5</td>
<td>14.42</td>
<td>AMP-dependent synthetase and ligase family protein</td>
</tr>
<tr>
<td>at1g23240</td>
<td>2.2</td>
<td>2.42</td>
<td>Acyl lipid metabolism: providing the core diffusion barrier of the membranes that separates cells and subcellular organelles.</td>
</tr>
<tr>
<td>at3g20520</td>
<td>2.4</td>
<td>2.86</td>
<td>Glycerol metabolic process</td>
</tr>
</tbody>
</table>
4.2.2 Secondary metabolism: Terpenoids

Many of the genes that were significantly downregulated in the polV and rdd mutants are related to the biosynthesis of secondary metabolites. Plants produce primary and secondary metabolites that encompass a wide variety of functions (Zwenger and Basu, 2008). Among these downregulated genes involved in the production of secondary metabolites are those encoding enzymes such as terpene synthases. Terpenes are produced in response to stress conditions, such as when a plant is activating its defense response against a pathogen (Keeling and Bohlmann, 2006). Volatile and non-volatile terpenes are implicated in the ability of plants to attract both pollinators and predators and in mediating thermotolerance and protection against photooxidative stress and microbes and insects. Our microarray data showed that several terpene synthase genes were significantly downregulated in the F. oxysporum-susceptible mutants polV and rdd (Table 4.2). This suggests that DNA methylation and demethylation pathways play a role in regulating the production of secondary metabolites and that the downregulation of these genes in the rdd and polV mutants may contribute to the observed hypersusceptibility to F. oxysporum infection.

Table 4.2 Commonly downregulated genes (terpene synthase) involved in secondary metabolism in rdd and polV mutants

<table>
<thead>
<tr>
<th>TAIR ID</th>
<th>rdd (fold change)</th>
<th>polV (fold change)</th>
<th>Related to</th>
</tr>
</thead>
<tbody>
<tr>
<td>at1g61680</td>
<td>6.6</td>
<td>17</td>
<td>Monoterpene biosynthetic process</td>
</tr>
<tr>
<td>at5g23960</td>
<td>13</td>
<td>31.55</td>
<td>Sesquiterpene biosynthetic process</td>
</tr>
<tr>
<td>at5g44630</td>
<td>3.8</td>
<td>5.85</td>
<td>Sesquiterpene biosynthetic process</td>
</tr>
<tr>
<td>at5g60510</td>
<td>2.09</td>
<td>2.32</td>
<td>Undecaprenyl pyrophosphate synthetase family protein</td>
</tr>
</tbody>
</table>
4.2.3 PR proteins

Pathogen-related (PR) proteins are proteins that are undetectable or barely detectable in healthy tissues but that accumulate in response to pathogen stress (Sels et al., 2008). An antifungal effect is an important common feature of most PR proteins. In addition, some PR proteins also exhibit antibacterial, insecticidal, nematicidal, and as recently shown, antiviral activities. The toxicity of PRs to fungal pathogens can generally be explained by their hydrolytic, proteinase-inhibitory, and membrane-permeabilizing characteristics, which are believed to weaken the fungal cell wall (Edreva, 2005). The commonly downregulated PR protein genes in *polV* and *rdd* are summarized in Table 4.3. This altered expression of PR protein genes could be one of the factors contributing to the compromised defense response to *F. oxysporum* in the susceptible mutants. This result suggests that both the DNA methylation and demethylation pathways regulate the expression of some PR genes and that these genes may be involved in antifungal defense.

Table 4.3 Commonly downregulated PR genes in *rdd* and *polV*

<table>
<thead>
<tr>
<th>TAIR ID</th>
<th><em>rdd</em> (fold change)</th>
<th><em>polV</em> (fold change)</th>
<th>Related to</th>
</tr>
</thead>
<tbody>
<tr>
<td>at2g15040</td>
<td>5.81</td>
<td>17.75</td>
<td>Disease resistance protein</td>
</tr>
<tr>
<td>at4g07820</td>
<td>4.25</td>
<td>2.80</td>
<td>CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 protein)</td>
</tr>
</tbody>
</table>
4.2.4 Cell wall-associated genes

The cell wall constitutes a physical barrier between the environment and the internal contents of a plant cell, and modifications of the cell wall are often associated with plant defense responses. Our microarray experiment identified several cell wall-associated genes that were significantly downregulated in both the polV and rdd mutants (Table 4.4).

Table 4.4 Cell wall-associated genes commonly downregulated in rdd and polV mutants

<table>
<thead>
<tr>
<th>TAIR ID</th>
<th>rdd (fold change)</th>
<th>polV (fold change)</th>
<th>Related to</th>
</tr>
</thead>
<tbody>
<tr>
<td>at1g02790</td>
<td>3.2</td>
<td>66.25</td>
<td>Carbohydrate metabolic process</td>
</tr>
<tr>
<td>at1g14420</td>
<td>4.37</td>
<td>6.63</td>
<td>Cell wall organization</td>
</tr>
<tr>
<td>at2g02720</td>
<td>2.73</td>
<td>3.86</td>
<td>Pectate lyase activity</td>
</tr>
<tr>
<td>at3g07820</td>
<td>3.45</td>
<td>22.31</td>
<td>Carbohydrate metabolic process</td>
</tr>
<tr>
<td>at2g47030</td>
<td>3.68</td>
<td>29.04</td>
<td>Cell wall modification</td>
</tr>
<tr>
<td>at2g26450</td>
<td>3.24</td>
<td>7.88</td>
<td>Cell wall modification</td>
</tr>
<tr>
<td>at2g47040</td>
<td>3.55</td>
<td>19.97</td>
<td>Pectin methylesterases</td>
</tr>
<tr>
<td>at3g17060</td>
<td>3.58</td>
<td>4.02</td>
<td>Pectinesterase activity</td>
</tr>
<tr>
<td>at3g05610</td>
<td>3.63</td>
<td>12.81</td>
<td>Pectinesterase activity</td>
</tr>
</tbody>
</table>
Cell wall-degrading enzymes (e.g., polygalacturonase) have been implicated in fruit softening during the ripening process (Hobson, 1963). In contrast, the transcript levels of various pectin methyl esterases (PMEs) are regulated by cold, wounding, ethylene exposure, and bacterial or viral infections (Lee and Lee, 2003; De Paepe et al., 2004). The expression patterns of specific clusters of *Arabidopsis* and *Populus* PMEs appear to correlate with specific biotic and abiotic stresses. Furthermore, altered plant susceptibility to pathogens and abiotic stresses has been associated with changes in PME activities in both PME antisense lines and in plants overexpressing PME or PME inhibitors, as well as changes in the degree of methylesterification of cell wall pectins (Pelloux et al., 2007; Volpi et al., 2011). The significant downregulation of PMEs observed in *polV* and *rdd* mutants could contribute to the disease susceptibility. Our results also suggest that these cell wall-associated genes might be regulated by the DNA methylation and demethylation pathways.

4.2.5 Auxin responsive factors

Emerging evidence suggests that auxin signaling differentially regulates resistance to different pathogens in plants (Kazan and Manners, 2009). In *Arabidopsis*, infection with *Botrytis cinerea*, a necrotrophic fungal pathogen, results in altered expression of the key genes involved in auxin signaling (Llorente et al., 2008). In particular, the infection process was shown to repress auxin response factors, thereby leading to increased susceptibility to the necrotrophic fungus (Llorente et al., 2008). The MapMan-based analysis of our microarray data showed that 70% of the auxin-related genes were downregulated in the *polV* mutant (Fig 4.1) but that 100% of these genes were downregulated in the *rdd* mutant (Fig 4.2). This result suggests that DNA methylation and
demethylation pathways might be involved in the regulation of auxin response genes and that these genes may play a role in the plant defense against *F. oxysporum*.

The microarray experiment was performed on uninfected wild-type and mutant *Arabidopsis* plants. The downregulation of biotic stress-related genes in uninfected *polV* and *rdd* plants implies that these mutant lines have a compromised capacity to defend against *F. oxysporum* infection prior to the fungal attack. However, to better understand the function of these genes in the development of *F. oxysporum* resistance, determining the expression patterns of these genes in the wild-type and mutant backgrounds following *F. oxysporum* infection will be important. Therefore, real-time RT-PCR (qRT-PCR) was performed to investigate the effect of *F. oxysporum* infection on the expression of some of the biotic stress-related genes that are downregulated in both *polV* and *rdd* mutants under normal conditions. The expression was examined at 1–2 days post-infection; the results are summarized in Table 4.5.
Table 4.5 qPCR analysis of the effect of *F. oxysporum* infection on the expression of biotic stress-related genes downregulated in both *polV* and *rdd* mutants under normal conditions

<table>
<thead>
<tr>
<th>Biotic stress-related genes</th>
<th>TAIR ID</th>
<th><em>polV</em></th>
<th><em>rdd</em></th>
<th><em>polV</em></th>
<th><em>rdd</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mock*</td>
<td>Mock#</td>
<td>*F. ox inf#</td>
<td>*F. ox inf#</td>
<td></td>
</tr>
<tr>
<td>at1g23240 (Lipid metabolism)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>at1g02790 (Cell wall)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>at3g07820 (Cell wall)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>at3g62230 (F-box protein)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>at1g61680 (Secondary metabolism)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>at2g15040 (Disease-resistance protein)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>at4g07820 (Pathogenesis-related protein)</td>
<td></td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

*Mock columns show the differential gene expression found in uninfected plants (treated with water) by microarray analysis.

# The *F. oxysporum* inf columns show the differential gene expression in plants infected with *F. oxysporum*, as found by qPCR.
Figure 4.7  qPCR measuring the level of at1g23240 relative to *FDH* in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.8 qPCR measuring the level of at3g07820 relative to FDH in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.9 qPCR measuring the level of at1g02790 relative to FDH in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.10 qPCR measuring the level of at3g62230 relative to FDH in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.11 qPCR measuring the level of at1g61680 relative to FDH in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.12 qPCR measuring the level of at2g15040 relative to FDH in *F. oxysporum-*infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.13 qPCR measuring the level of at4g07820 relative to FDH in *F. oxysporum*-infected wild-type, *polV* and *rdd* mutant plants at 1-2 days post infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
As shown in Fig 4.7 to Fig 4.13, all of the genes tested in the polV mutant remained downregulated relative to the wild type, following F. oxysporum infection. We observed a similar trend in the rdd mutant, with the exception of a secondary metabolite-associated gene (at1g61680), which was upregulated relative to the wild type following infection. This suggests that overall the tested biotic stress-related genes were not induced in either polV or rdd mutants following F. oxysporum infection.

We also analyzed the expression of pathogenesis-related 1 (PR1; at2g14580), a marker for an intact salicylic acid (SA) pathway (Thomma et al., 1998; Van Loon and Van Strien, 1999). The expression level of PR1 was higher in the mock-treated polV mutant plants than in the wild-type plants (Fig 4.14 a). Furthermore, experiments were performed to analyze the expression of PR1 following F. oxysporum infection, and the results of this experiment are described below.

4.2.6 The SA pathway is compromised in polV and rdd mutants following F. oxysporum infection

The role of the SA pathway in plant defense has been well documented. I have experimentally validated our microarray data with RT-PCR, showing that the basal expression of PR1 was indeed upregulated in the mock-treated polV mutants (Fig 4.14b). To further investigate the expression of PR1 in susceptible mutants, its expression pattern following F. oxysporum infection was examined. RT-PCR analysis showed detectable level of PR1 expression following F. oxysporum infection in the wild-type plants at 1 dpi (Fig 4.15). In contrast, PR1 expression in polV and rdd mutants was undetected or lower as compared to the wild-type plants at both 1 and 3 dpi (Fig 4.15); however, at 6 dpi, PR1 expression in polV and rdd mutants was equivalent to
the level seen in the wild-type plants (Fig 4.15). These results indicate that the SA pathway in the susceptible mutants is impaired only during the early stages of *F. oxysporum* infection.
Figure 4.14 a PR1 expression in uninfected wild-type and polV mutant plants, as depicted in our microarray analysis. b Experimental validation of PR1 expression by using RT-PCR analysis. R1 and R2 = 2 technical replicates. The housekeeping gene actin was equally amplified in all samples.
Figure 4.15 PR1 expression in *F. oxysporum*-infected wild-type, *polV*, and *rdd* mutant plants. RT-PCR analysis shows the amplification of *PR1* and the housekeeping gene *FDH* in *F. oxysporum*-infected wild-type plants and *polV* and *rdd* mutant plants at 1, 3, and 6 dpi.
4.2.7 Induction of \textit{AtWRKY70} and \textit{AtWRKY33} requires RdDM and demethylation components upon pathogen infection

More than 70 WRKY transcription factors are encoded in the \textit{Arabidopsis} genome, and many are induced during plant defense (Eulgem and Somssich, 2007). Previous studies have shown the involvement of \textit{AtWRKY70} and \textit{AtWRKY33} in plant defense against bacterial and fungal pathogens (Li et al., 2004; Knoth et al., 2007; Ulker et al., 2007). In this chapter, I have expanded the expression analysis to other key defense-related genes that were implicated in plant defense and epigenetically regulated following pathogen infection. In this chapter, the role of methylation and demethylation factors in regulating the expression of \textit{AtWRKY70} and \textit{AtWRKY33} was elucidated.

To investigate the role of methylation and demethylation factors in this process, we analyzed the expression of \textit{AtWRKY70} and \textit{AtWRKY33} 1 day after \textit{F. oxysporum} infection in wild-type, \textit{polV}, and \textit{rdd} plants. The expression of \textit{AtWRKY70} and \textit{AtWRKY33} was found to be downregulated in the \textit{polV} and \textit{rdd} mutants at 1 day post-infection relative to the wild-type plants (Figs. 4.16 and 4.17). This suggests that induction of \textit{AtWRKY70} and \textit{AtWRKY33} to wild-type levels requires PolV and RDD factors following \textit{F. oxysporum} infection.
Figure 4.16 Relative expression of *WRKY70* in mock-treated and *F. oxysporum*-infected wild-type, *polV*, and *RDD* mutant plants at 1 day post-infection. Bars represent the average relative concentration determined using three technical replicates. Error bars represent the standard deviation of the replicates.
Figure 4.17 Relative expression of WRKY33 in mock-treated and \textit{F. oxysporum}-infected wild-type, \textit{polV}, and \textit{rdd} mutant plants 1 day post-infection. RT-PCR analysis shows the amplification of \textit{WRKY33} and the housekeeping gene \textit{FDH} in mock-treated and \textit{F. oxysporum} infected wild-type, \textit{polV}, and \textit{rdd} mutant plants at 1 day post-infection.
4.3 Discussion

In chapter 3, I have described the susceptible phenotype of polV and rdd mutant plants following *F. oxysporum* infection. The result from *F. oxysporum* infection suggested that PolV and demethylases may play a key role in plant defense against *F. oxysporum* by regulating defense-related genes. We therefore analysed gene expression in these mutants relative to the wild-type using microarray technique.

The microarray analysis showed that a significant number of genes associated with cell wall synthesis, secondary metabolism, and disease resistance were downregulated in the *polV* mutant relative to the wild-type. This suggests that PolV plays a positive role in the expression of these genes under normal conditions.

In *Arabidopsis*, it is well established that PolV facilitates transcriptional silencing of transposable elements, repeat elements, pseudogenes, overlapping genes, and adjacent genes by virtue of cytosine hypermethylation and other repressive chromatin modifications such as H3K9 and H3K27 methylation (Bernatavichute et al., 2008; Fuchs et al., 2006; Gendrel et al., 2002; Mathieu et al., 2005; Turck et al., 2007).

It was previously hypothesized that PolV is required for facilitating transcription throughout the genome at both silenced and non-silenced regions (Wierzbicki et al., 2008). PolV transcripts are detectable in transposon-rich, heterochromatic regions as well as in gene-rich, presumably euchromatic regions (Wierzbicki et al., 2008). Therefore, it is plausible that PolV or PolV-dependent transcripts may be involved in initiating or assembling the protein complex required
for maintaining the expression of those genes that were shown to be downregulated in our microarray studies.

Another possible explanation for the widespread down-regulation of genes in the polV mutant could be the down-regulation of demethylase ROS1. Our microarray data showed that ros1 was significantly downregulated (~4-fold) in the polV mutant relative to the wild-type. ROS1 removes methylation in CG and non-CG contexts; therefore, the down-regulation of ROS1 in polV mutants, which is compromised in non-CG methylation, may have resulted in the hypermethylation of genes particularly at CG sites, resulting in their suppression.

This down-regulation of ROS1 in polV mutants as observed in our microarray study is consistent with previous findings where ROS1 was shown to be transcriptionally downregulated in the plants with mutations in RDR2, DRD1, and PolIV genes (Huettel et al., 2006). It was suggested that the effect on ROS1 expression appears to be specific to the mutations that disrupt the PolIV/RDR2/DCL3/AGO4 pathway, because mutations in RDR6 do not affect ROS1 expression. ROS1 was also downregulated in the plants with mutations in the de novo methyltransferase DRM2 (Penterman et al., 2007). Since RDR2, DRD1, PolIV, and DRM2 all encode enzymes that function at different, critical steps of the PolIV/RDR2/DCL3/AGO4 pathway (Li et al., 2006; Pontes et al., 2006), it has been suggested that the function of the pathway as a whole, rather than of any one component, is important for ROS1 expression. ROS1 down-regulation in the RdDM mutants may be required to counterbalance the reduction in DNA methylation. However, this counterbalance mechanism may cause hypermethylation in the CG
context (Penterman et al., 2007), which on similar lines could account for the down-regulation of some of the genes in the polV mutant.

As in polV, we have shown that many genes involved in biotic stress were also downregulated in the rdd mutant. This down-regulation could be attributed to the hypermethylation of genes in the CG and non-CG contexts, since DML2, DML3, and ROS1 erase methylation marks in both sequence contexts (Penterman et al., 2007). In a previous study, 179 loci with increased methylation were identified in the rdd mutant relative to the wild-type controls. These loci were enriched for transposons, repetitive DNA elements, and siRNA generating loci; ~80% were close or overlapping annotated genes, and the increase in DNA methylation was primarily located at the 5’ and 3’ ends of the genes, indicating that demethylase enzymes act at both normally silenced loci (i.e., transposons) and the boundaries between euchromatin and heterochromatin (i.e., the genes residing in or near heterochromatic environments) (Penterman et al., 2007; Law and Jacobsen 2010). At such boundaries, these glycosylases may protect the RdDM-targeted genes. Moreover, various defense-related genes are clustered in transposable element (TE)-rich areas or repeat-rich regions that are frequently targeted by the RdDM pathway (Alvarez et al., 2010, Bernatavichute et al., 2008). Therefore, it is plausible that the spread of DNA methylation from the TE/repeat-rich regions to adjacent or overlapping genes may have resulted in the hypermethylation of genes in the CG and non-CG context in rdd mutant plants. This suggests that under normal conditions, the expression of a large number of genes, including those associated with biotic stress, is maintained at wild-type levels by the components of the methylation and demethylation machinery, underlining the importance of these epigenetic mechanisms in plant growth, development, and immunity.
Among the upregulated genes in the polV mutant, we found a number of transposons and repeat elements, consistent with the role of PolV in facilitating de novo methylation at these elements. Interestingly, our microarray data showed the up-regulation of PR1 (a marker for intact SA defense-signaling pathway) in the polV mutant compared to the wild-type under normal (uninfected) conditions. This finding is consistent with the previous findings showing similar up-regulation of PR1 gene expression in chromatin remodeling mutants such as brm101 (Brahma), sni1 (suppressor of NPR1, inducible 1), and pie-5 (Photoperiod-Independent Early Flowering 1) (Bezhani et al., 2007; Mosher et al., 2008; Rosana et al., 2008), and it reinforces the observation that the SA pathway is negatively regulated by the epigenetic factors including PolV in the wild-type plants under normal conditions.

We tested PR1 expression at 1 and 3 days following F. oxysporum infection, and found that PR1 was significantly downregulated compared to the wild-type control in the susceptible mutants polV and rdd, although PR1 expression returned to the wild-type levels at 6 dpi. Previously, it was shown that pathogens such as Botrytis cinerea and Alternaria brassicicola produce toxins that interfere with plant chromatin or the chromatin modification machinery to suppress the expression of plant defense genes. The host selective virulence factor HC toxin, produced by some strains of Cochliobolus carbonum, inhibits host histone deacetylases, and thus suppresses elicitor-activated defense in maize (Zea mays) (Brosch et al., 1995; Ransom and Walton, 1997; Wight et al., 2009).

SA pathway is involved in imparting resistance to biotrophs. F. oxysporum is considered a hemibiotroph pathogen that begins its life cycle on living tissue as a biotroph, and then completes its life cycle on dead tissue as a necrotroph (Agrios, 2005). Given the initial down-regulation of
PR1 following *F. oxysporum* infection in susceptible mutants, it is possible that this pathogen may modulate the expression of defense-related genes like as PR1 in order to establish the initial infection by suppressing the SA pathway. This, in turn, would favor the growth of the pathogen and help establish a successful infection.

In a recent study, it was found that the *polV* mutant accumulates higher levels of H3k4me3 on the PR1 promoter under normal conditions; however, no DNA methylation was observed on the PR1 promoter of the wild-type (Lopez et al., 2011). This lack of a DNA methylation in the DNA of the defense-related PR1 indicates that the differential expression of PR1 is not due to the altered DNA methylation pattern resulting from a defective RdDM pathway, but instead may be due to the differential modification of histones (Lopez et al., 2011). Thus, *F. oxysporum* may have caused the dismantling and assembling of histone proteins, leading to early down-regulation and delayed induction of PR1 in the *polV* and *rdd* mutants following *F. oxysporum* infection.

Previous studies suggest that during environmental stresses, the early signaling events determine whether the plants can cope with the condition (Smith and Stitt, 2007; Baena-González and Sheen, 2008). It was previously shown that the largest transcriptional differences triggered by salt stress in a tolerant versus a sensitive rice variety occurred within the first hour of exposure. Failure to regulate the appropriate genes at the appropriate time ultimately resulted in cell death in the salt-sensitive variety (Kawasaki et al., 2001).
It is, therefore, possible that the down-regulation of defense-related genes such as PR1 during the early stages of *F. oxysporum* infection, along with failed induction of other defense-related genes, including some biotic stress-related genes, contributes to the increased susceptibility of *polV* and *rdd* mutants to *F. oxysporum* infection.

Moreover, it has been shown that transgenic expression of tobacco PR1 increased the resistance to *F. oxysporum* in an *Arabidopsis* mutant (*esa1*) shows enhanced susceptibility to pathogen attack (Van Hemelrijck et al., 2006). Plants expressing an SA-degrading enzyme are more susceptible to *F. oxysporum* (Gaffney et al., 1993). Hence, it is likely that down-regulation of various defense-related genes, including PR1, contribute to the susceptible phenotype of *polV* and *rdd* mutants.

Preliminary experiments in our lab indicate that 1–3 days after *F. oxysporum* infection, the wild-type plants show higher methylation levels than the untreated wild-type plants, suggesting that the infected wild-type plants accumulate more DNA methylation under stress conditions. This is consistent with previous studies on F1 maize hybrids and their parents, which showed that under dense planting (a stress condition), the parents accumulated more DNA methylation sites than their hybrids, which are resistant to DNA methylation changes (Kovacevic et al., 2005; Tani et al., 2005; and reviewed in Tsaftaris et al., 2008). Interestingly, various studies have indicated a role for demethylation under stressful conditions to counterbalance the deleterious effects of methylation. Tobacco plants exposed to high concentrations of salt and aluminum and cold temperatures displayed changes in the methylation pattern of a gene encoding glycerophosphodiesterase-like protein (NtGPDL), which is known to be induced in response to
aluminum stress (Choi and Sano, 2007). CG sites within the coding region were selectively demethylated, suggesting that abiotic stress caused gene activation by changing the DNA methylation status of the particular genomic locus. A recent study exploring the genome-wide DNA methylation status of two rice cultivars with different tolerance to drought revealed significant differences in the methylation patterns between the 2 genomes (Wang et al., 2011). Changes in DNA methylation/demethylation were induced under drought conditions in a developmental and tissue-specific manner, and they accounted for 12.1% of the total site-specific methylation differences between the 2 lines. Notably, 70% of the drought-induced methylation changes were reversed after recovery, while only 29% remained unaltered. These observations suggest that DNA methylation/demethylation changes play a role in the response to stress conditions, probably by activating or deactivating stress-responsive genes, thereby leading to better adaptation of a plant to unfavorable conditions (Wang et al., 2011). Therefore, after the initial stress-induced down-regulation of PR1 at days 1 and 3 following F. oxysporum infection, activation of PR1 in polV and rdd mutants may have occurred by virtue of histone modifications to restore the expression of PR1 to the wild-type levels in order to help the cells deal with the stress of pathogenic infection.

We also investigated the expression of AtWRKY70 and AtWRKY33 transcription factors which are known to be involved in plant defense against bacterial and fungal pathogens, following F. oxysporum infection AtWRKY70 is known to activate the SA pathway and positively regulates the expression of the PR1 gene (Li et al., 2004). We have shown that the expression of AtWRKY70 is downregulated in polV and rdd mutant plants at 1 day following F. oxysporum
infection. This down-regulation of AtWRKY70 is positively correlated with the down-regulation of PRI in polV and rdd mutants at 1 day post infection.

A previous report indicates that methylation of H3K4 at the nucleosomes of WRKY70 stimulates the SA-dependent defence responses (Álvarez-Venegas et al., 2007). In wild-type plants, infection with P. syringae induces the expression of WRKY70 which is associated with the reduction of H3K27me2 and the accumulation of H3K4me2 and H3K4me3 at WRKY70 nucleosomes. Importantly, the modifications found at WRKY70 nucleosomes in infected plants are associated with the activity of Arabidopsis Trithorax 1 (ATX1), a SET-domain protein that acts as an H3K4 methylase (Alvarez-Venegas et al., 2003). Infected atxl mutant plants show weak activation of WRKY70 and, in these plants WRKY70 nucleosomes lack H3K4me3 but contain H3K27me2 and H3K4me2 levels comparable with those of infected wild-type plants. Thus, transcriptional activation of WRKY70 is induced by ATX1 through apparent trimethylation of H3K4 (Álvarez-Venegas et al., 2007). Like the requirement of ATX1 for AtWRKY70 induction and accumulation of H3K4me2 and H3K4me3 in wild-type plants, it is possible that PolV and RDD factors are required for the induction of AtWRKY70 to the wild-type levels following F. oxysporum infection. Thus, mis-expression of WRKY70 following infection in the polV and rdd mutant background suggested additional epigenetic routes for its control.

AtWRKY33 is downregulated following F. oxysporum infection in polV and rdd mutants at 1 day post infection. A previous report suggests that mutations of the Arabidopsis WRKY33 gene encoding a WRKY transcription factor cause enhanced susceptibility to the necrotrophic fungal pathogens Botrytis cinerea and Alternaria brassicicola. Ectopic over-expression of WRKY33, on the other hand, increases resistance to the two necrotrophic fungal pathogens (Zheng et al.,
Recently, global expression profiling on susceptible *wrky33* and resistant wild-type plants uncovered massive differential transcriptional reprogramming upon *B. cinerea* infection at 14 hours post inoculation. Genes involved in redox homeostasis, SA signaling, ethylene (ET)-JA mediated cross-communication, and camalexin biosynthesis were identified as direct targets of WRKY33 (Birkenbihl et al., 2012). Based on these observations the compromised induction of *AtWRKY33* in *polV* and *rdd* mutants relative to the wild-type may lead to mis-expression of genes thereby contributing to the diseased phenotype of susceptible mutants.

Taken together, the expression data in this study suggest that methylation and demethylation factors regulate a large number of genes, many of which are involved in biotic stress responses. The microarray analysis was done with uninfected plants, and the expression of only a small number of genes was analyzed in infected tissues using RT-PCR techniques. Further expression analyses should be devoted to *F. oxysporum*-infected *polV* and *rdd* mutants, and should be performed in a time course-based manner to better understand *F. oxysporum* modulated genes and their function in the development of resistance against *F. oxysporum*.
CHAPTER 5: Role of AGO4 in antifungal defense against *Fusarium oxysporum*
5.1 Introduction

The influence of epigenetic regulation on control of the adaptive responses of living organisms to changes in the environment is becoming a common theme in biology. RNA–directed DNA methylation (RdDM) is an epigenetic control mechanism driven by a subset of non-coding small interfering RNAs (siRNAs) which can influence gene function without altering the DNA sequence of their target genomic regions, either by inducing de novo methylation of cytosine residues or by modifying histones (Matzke et al., 2009; Law and Jacobsen, 2010). The implications and roles of the RdDM pathway in the orchestration of plant immune responses still remain to be fully characterized.

Increasing evidence also indicates that endogenous plant small RNAs, including microRNAs (miRNAs) and siRNAs, are integral components of the machinery which regulates plant defense against microbial pathogens (Katiyar-Agarwal et al., 2010). Arabidopsis miR393 imparts basal resistance to the bacterial pathogen Pseudomonas syringae DC3000 (PsDC3000) by targeting the auxin receptors TIR1, ABF2 and ABF3 (Navarro et al., 2006). Similarly, Lu et al. identified a series of 10 miRNA families in loblolly pine whose expression were suppressed upon infection with the rust fungus Cromartium quercuum f. sp. fusiforme, which subsequently lead to increases in the expression levels of the target genes of these miRNA families (Lu et al., 2007). Likewise, miR1885 is upregulated upon infection of Brasica rapa with Turnip mosaic virus (TuMV), and the target of miR1885 is predicted to be a member of the nucleotide-binding site leucine-rich repeat (TIR-NBS-LRR) class of disease-resistance proteins (He et al., 2008). Plants contain only several hundred miRNAs and large numbers of endogenous siRNAs; however, only a few cases describing the involvement of siRNAs in plant immunity have been reported. For
example, the natural antisense transcript (NAT)-derived nat-siRNAATGB2 and long siRNA 1 (isiRNA-1), which specifically target the mitochondrial pentatricopeptide protein (PPR)-like gene PPRL and the RNA-binding protein gene (AtRAP), respectively, are induced by the bacterial pathogen PsDC3000 (avrRpt2) in Arabidopsis and contribute to plant antibacterial immunity (Katiyar-Agarwal et al., 2006). Another example of siRNA-mediated plant resistance responses are the endogenous siRNAs generated at the disease resistance (RPP4) locus, which impart resistance to both the bacteria P. syringae pv. maculicola and the oomycete Hyaloperonospora arabidopsidis (Yi and Richards, 2007).

The Arabidopsis ago4-1 mutant was first discovered in a screen for mutants that suppress silencing of the SUPERMAN (SUP) gene (Zilberman et al., 2003). Analysis of ago4-1 indicated that these mutants contained reduced levels of non-CG methylation as well as methylation of histone H3 lysine-9 in: the SUP gene, the MEA-ISR intergenic locus, and AtSN1 repetitive elements. Arabidopsis AGO4 and associated sRNAs are important for maintaining the transcriptionally silent state of heterochromatic regions, repetitive sequences, and transposable elements (Tran et al., 2005). Previous studies in the model plant Arabidopsis have revealed that ARGONAUTE4 (AGO4), one of the characteristic components of the RdDM pathway, is required for plant immunity against bacterial pathogens (Agorio and Vera, 2007). There have been no previous studies reporting the involvement of AGO4 in Fusarium wilt disease caused by F. oxysporum. In this chapter, the possible role of AGO4 in plant resistance to F. oxysporum is explored.
5.2 Results

5.2.1 Expression of AGO4 is affected in polV and rdd mutants upon Fusarium oxysporum infection

In Arabidopsis, AGO4 is shown to impart resistance against bacterial pathogen P.syringae and fungal pathogens B.cinerea and P.cucumerina (Agorio and Vera, 2007; Lopez et al., 2011). To determine whether the impaired disease resistance in the susceptible mutants polV and rdd in response to F.oxysporum is associated with misregulation of AGO4, we analyzed the expression of AGO4 at 1 and 3 day following F.oxysporum infection in the wild-type, polV and rdd plants. We found that the expression of AGO4 was strongly downregulated in polV and rdd mutants at 1 day post infection relative to the wild-type plants (Fig. 5.1). This down-regulation of AGO4 was more pronounced in the rdd mutant than that of polV and wild-type.

Next, we analysed the expression of AGO4 at 3 days post infection and we found, that the expression of AGO4 was further reduced in polV mutant plants at 3 days post infection relative to the wild-type plants (Fig. 5.1). Surprisingly, in rdd mutant plants, the AGO4 expression was drastically down almost undetectable relative to the wild-type plants.
Figure 5.1 Northern blot analysis detecting AGO4 transcript in mock (M) treated and infected (F) wild-type, polV, and rdd plants 1 and 3 day post infection. Corresponding loading controls are shown below.
5.2.2 AGO4 is required for antifungal defense against *F. oxysporum* in *Arabidopsis*

In order to dissect the role of AGO4 in plant defense against fungal pathogen, we assayed the *Arabidopsis de novo* methylation pathway mutant *ago4* for altered resistance or susceptibility to *F. oxysporum*.

In the first series of experiments, 4 week-old wild-type, *ago4* and *rdd* (internal control) plants were grown in soil and then inoculated with *F. oxysporum* and the disease symptoms were evaluated at 10 days post inoculation (dpi). Mock-treated *ago4* and *rdd* plants did not display any visible developmental defects or abnormalities and looked similar to mock-treated wild-type plants (figure not shown). At 10 dpi, *ago4* mutant plants displayed a diseased phenotype with moderate vein clearing and chlorosis of the leaves, in contrast to wild-type plants where a mild yellowing was only observed in some leaves (Fig. 5.2a). At this stage, as expected, *rdd* mutant plants displayed more severe yellowing of the leaves than the *ago4* mutants and the wild-type plants.

Next, a sugar-free MS agar based assay system was used to screen altered phenotype of *ago4* mutant plants in response to *F. oxysporum* infection. Four week-old wild-type, *ago4*, and *rdd* plants were infected with *F. oxysporum* on sugar-free MS plates. Consistent with the results of the soil infection experiment, the *ago4* mutant plants displayed stronger disease symptoms such as severe and more prominent vein clearing of the leaves than wild-type plants at 10 dpi (Fig. 5.2b). At this stage, *rdd* exhibited extensive necrosis and yellowing of the leaves than the *ago4* and wild-type plants.
Figure 5.2 *Arabidopsis* demethylation (*rdd*) and *de novo* methylation (*ago4*) mutants display increased susceptibility to *F. oxysporum*. Four week old wild-type, *rdd* and *ago4* plants were inoculated with *F. oxysporum* (10^7 spores/ml) and the disease severity was assessed 10 days after inoculation. **a.** *F. oxysporum* infected plants in soil; **b.** *F. oxysporum* infected plants on MS agar.
5.2.3 Overexpression of AGO4 improves tolerance to *Fusarium oxysporum*

As shown in the previous section, the loss-of-function phenotypes of *ago4* mutant in response to *F. oxysporum* indicate that AGO4 is a critical factor in defense response against this pathogen. To further investigate the role of AGO4 in disease resistance against *F. oxysporum* full-length cDNA fragment of *AGO4* were over-expressed in *Arabidopsis C 24 ecotype* driven by the cauliflower mosaic virus 35S promoter (construct kindly provided by Dr Chris Helliwell).

A sugar-free MS agar based assay system was used to screen independent transgenic lines (provided by Dr Chris Helliwell) over-expressing AGO4 protein for *F. oxysporum*-resistant or susceptible phenotypes. Three week-old wild-type plants over-expressing AGO4 protein were infected with *F. oxysporum* and analysed for disease resistance or susceptibility at 10 days post infection. We found, in our analysis that transgenic lines 35S-AGO4-#5, 35S-AGO4-#7, 35S-AGO4-#8 (Fig. 5.3) displayed more tolerant disease phenotypes to *F. oxysporum* as compared to the non transgenic wild-type plants. On the other hand transgenic lines 35S-AGO4-#1, 35S-AGO4-#4 did not show any resistant phenotype to *F. oxysporum*. The Western –blot (Fig. 5.4) revealed that the tolerant disease phenotype of transgenic lines 35S-AGO4-#5, 35S-AGO4-#7, 35S-AGO4-#8 (Fig. 5.3) was well correlated with higher AGO4 protein levels whereas 35S-AGO4-#1, 35S-AGO4-#4 did not show any resistant phenotype to *F. oxysporum* which was again correlated well with reduced level of AGO4 protein in these transgenic lines. This suggests that in order to rescue a diseased phenotype a minimal level (threshold) of AGO4 protein is required for disease resistance.

Notably, transgenic line 35S-AGO4-#1, showed low level of AGO4 protein. It is likely that this transgene may have integrated into heterochromatic regions of plant genomes which tend to be transcriptionally silenced (Schubert et al., 2004); a phenomenon known as the position effect.
Figure 5.3 *Arabidopsis* transgenic lines (wild-type C-24 ecotype) over-expressing AGO4 protein showing various degree of disease symptoms to *F. oxysporum*. Three week old plants were inoculated with *F. oxysporum* (10⁷ spores/ml) and the disease severity was assessed 10 days after inoculation on MS agar plates.
Figure 5.4 Western blot showing varied levels of AGO4 protein in transgenic Arabidopsis over-expressing an AGO4 transgene.
5.3 Discussion

In Chapter 3, the *F. oxysporum* susceptible phenotype of *polV* (methylation) and *rdd* (demethylation) Arabidopsis mutants was characterized. As AGO4 physically interacts with PolV in the RdDM pathway, and AGO4 has previously been shown to be involved in antibacterial defense against *P. syringae* (Agorio and Vera, 2007), we hypothesized that AGO4 may play a role in the disease susceptibility of the *polV* and *rdd* mutants in response to *F. oxysporum*. Thus, the role of AGO4 in the disease susceptibility of *polV* and *rdd* mutants to *F. oxysporum* was explored in this chapter.

I analysed the expression of AGO4 in wild-type, *polV* and *rdd* plants before and after *F. oxysporum* infection. Expression analysis revealed that AGO4 was downregulated in the *F. oxysporum* susceptible *polV* and *rdd* mutants at 1 and 3 days post infection, relative to wild-type plants. The down-regulation of AGO4 was specifically induced by *F. oxysporum* infection, and was not due to the presence of the *polV* or *rdd* mutations, as Northern blotting and microarray analysis did not indicate any down-regulation of AGO4 in mutant plants relative to wild-type under normal conditions. Previously, it was shown that virulence proteins directly modulate plant chromatin remodeling via HC-toxin produced by the maize (Zea mays) fungal pathogen Cochliobolus carbonum. HC-toxin inhibits histone deacetylase activity, leading to hyperacetylation of histones during infection (Brosch et al., 1995; Chen and Tian, 2007; Walton, 2006; Yang and Seto, 2008) which ultimately leads to maize corn leaf disease. Thus, the down-regulation of AGO4 in infected plants suggests that *F. oxyporum* toxins or effectors may have modulated the expression of AGO4, in order to establish successful infection.
Moreover, pathogen induced up or down-regulation of chromatin associated genes has been reported before (Li et al., 2010; Clough et al., 2000; Jurkowski et al., 2004; Kim et al., 2008; Zhu et al., 2010; Wang et al., 2010). Therefore, it is plausible that *F. oxysporum* may have induced repression of key defense genes such as *AGO4* and *PRI* (shown in Chapter 4) via histone modifications or DNA methylation, as a part of the infection process.

The interaction between DNA methylation and demethylation has been reported before (Penterman et al., 2007; Zheng et al., 2008). The RdDM pathway is known to regulate the expression of ROS1 (demethylase enzyme). The down regulation of ROS1 is observed in various RdDM mutants including *polV* under normal conditions.

Thus, it is likely that the down-regulation of *AGO4* following *F. oxysporum* infection in *polV* mutant relative to wild-type plants may further contribute to continued *ROS1* down-regulation which may compromise the ability of these plants to demethylate disease resistance genes, such as *AGO4* following *F. oxysporum* infection. In contrast, the *rdd* triple mutant is compromised in demethylase enzymes and has a severely compromised ability to demethylate defense-related genes; therefore, severely susceptible phenotype of *rdd* is evident following *F. oxysporum* infection.

Furthermore, in Chapter 4, I have shown that most of the stress related genes which we tested were failed to be induced following *F. oxysporum* infection in susceptible mutants during early infection. Thus, continued repression of ROS1 seems plausible under pathogen stress during
early stages of infection which would favor disease development triggered by \textit{F. oxysporum} by virtue of establishing repressive modifications on genes targeted by demethylases.

AGO4 is one of the critical components in the transcriptional gene-silencing pathway associated with siRNA that directs DNA methylation of genes and pseudogenes (Qi et al., 2006; Agorio and Vera, 2007). Thus, the down-regulation of \textit{AGO4} in susceptible mutants \textit{polV} and \textit{rdd} may therefore lead to the activation of loci’s targeted by AGO4 associated small RNAs. Hence, it is possible that the undesirable genes or the genes that impart susceptibility which were silenced by TGS under normal conditions in the wild-type plants may become activated (release of TGS) by the \textit{AGO4} down-regulation in susceptible mutants following \textit{F. oxysporum} infection.

Likewise, other key defense related genes may become repressed due to compromised function of demethylase enzymes in \textit{polV} and \textit{rdd} mutants relative to the wild-type. Thus it is likely that this global imbalance between the induction and repression of several genes before and after the infection resulted from \textit{AGO4} down-regulation and \textit{polV, rdd} mutations, lead to defective defense response. Furthermore, \textit{ago4} mutant was found to be susceptible to necrotrophic pathogens \textit{Botrytis cinerea} and \textit{Plectosphaerella cucumerina} whereas \textit{ddm1} mutant plants (DDM1 is required to maintain DNA methylation), were found to be susceptible biotrophic oomycete pathogen \textit{Hyaloperonospora arabidopsidis} Noco2 (Lopez et al., 2011; Li et al., 2010). These observations further outline the importance of epigenetic factors in plant defense.

Additionally, our inoculation experiments revealed less severe diseased phenotype of \textit{ago4} mutant following \textit{F. oxysporum} when compared to the diseased phenotype of \textit{polV} and \textit{rdd} (as
shown in Chapter 3 and 5). AGO4 shows overlapping functions with AGO6 (Zhang et al., 2007). Thus, loss of AGO4 function would not necessarily lead to loss of non-CG methylation, if AGO6 could bind the same sRNAs or target the same genomic loci. Therefore, it is possible that the role of AGO4 may have been compensated by other members of the AGO family such as AGO6. whereas PoIV and RDD enzymes are unique and their role may not be compensated by other factors.

Taken together, our observations reveal that AGO4 may be a key defense-related gene which possibly plays multiple roles in plant defense against fungal and bacterial pathogen, in addition to its role in the RdDM pathway.
CHAPTER 6: Conclusion

Recent evidence indicates that RNA silencing plays a role in plant defense against not only viruses but also bacterial pathogens, and that bacteria have developed mechanisms to suppress RNA silencing to cause diseases (Navarro et al., 2006). However, how RNA silencing mechanisms are involved in the interaction between plants and non-viral pathogens remains largely unknown.

This project aimed at identifying RNA silencing-associated factors that are involved in DNA methylation and demethylation and important in plant defense against the fungal pathogen *F. oxysporum*.

We screened *Arabidopsis* mutants deficient in DNA methylation and demethylation factors for susceptibility or resistance to *F. oxysporum* to examine if DNA methylation and demethylation play a role in plant resistance to *F. oxysporum*. These experiments identified the RNA-directed DNA methylation (RdDM) factor PolV and the demethylases ROS1, DML2 and DML3 as critical components in Fusarium resistance; *polV* and *rdd* mutants were highly susceptible to this pathogen in comparison to wild-type *Arabidopsis*.

We also investigated whether susceptibility of *polV* and *rdd* mutant plants to *F. oxysporum* was conferred in the roots or shoots. *F. oxysporum* infects plants via the roots; therefore, determining whether the roots play a major role in the phenotypic response of *rdd* and *polV* mutants to *F. oxysporum* is important. We used a grafting technique to study the role of roots and shoots in the plant response to *F. oxysporum*. Our grafting experiment shows that reciprocal grafts between wild-type and susceptible mutants (*polV* or *rdd*) displayed an intermediate phenotype in response to *F. oxysporum* infection, suggesting that resistance to *F. oxysporum* occurs at the whole plant level and is mediated by both the roots and shoots in *Arabidopsis*. 
In order to understand how the identified RNA silencing factors are involved in plant defense against *F. oxysporum*, a genome-wide microarray-based study was performed. Based on the microarray analysis, we found that a number of genes associated with cell wall synthesis, secondary metabolism, transcription factors and hormone signaling pathways were down-regulated in the *polV* and *rdd* mutants relative to the wild-type, suggesting a specific role or requirement of PolV and the demethylases at these loci for maintaining gene expression under normal conditions. To gain further insight, we performed expression analysis before and after *F. oxysporum* infection on selected genes which were affected in *polV* and *rdd* mutant plants under normal conditions as shown in our microarray data. Our results show that various biotic stress related genes that were down-regulated in *polV* and *rdd* mutants under normal conditions remained down-regulated following *F. oxysporum* infection.

In addition, our microarray data revealed that the expression level of *PRI* was not reduced and even higher in the *polV* plants than in the wild-type plants under normal conditions. However, following *F. oxysporum* infection, the expression of *PRI* was significantly down-regulated in the *polV* and *rdd* mutants, indicating the involvement of RdDM and DNA demethylases in modulating the expression of key defense related genes such as *PRI* under fungal infection. It is interesting to note that after the initial stress-induced down-regulation of *PRI* at days 1 and 3 following *F. oxysporum* infection in *polV* and *rdd* mutants, *PRI* expression was reactivated at day 6 after infection. This reactivation occurred in the absence of the three demethylases (although the demethylase DME is still present but it is thought to function only in the seed), which implies that histone modifications (in addition to DNA methylation) may also be involved in the control of this gene (Lopez et al., 2011).
Our microarray data showed that *ros1* was significantly down-regulated (~4-fold) in the *polV* mutant relative to the wild-type. ROS1-mediated active demethylation and the RdDM pathway have opposing functions but have an interesting inter-dependent relationship (Penterman et al., 2007). In the absence of functional RdDM such as in the *polV* mutant, active demethylation is compromised and possibly preventing up-regulation of disease resistance genes, and this could (partly) account for the disease susceptibility of the *polV* mutant. ROS1 is also required for normal tolerance to genotoxic agents and DNA repair, and the absence of this functional enzyme in the *rdd* mutant likely results in increasing the susceptibility of plants cells to intra- and intercellular toxins that damage DNA (Gong et al., 2002).

A large number of genes showed similar patterns of altered expression in *polV* and *rdd* mutant plants relative to the wild-type. This raised the question of how these seemingly antagonistic factors PolV and DNA demethylases could possibly modulate the expression of number of genes in the same direction. In addition, that PolV and DNA demethylases can both activate and repress expression raises further questions of how this is achieved. One possible scenario is that mis-regulated genes such as transcription factors (TFs) are primary targets of epigenetic modifiers, while downstream genes controlled by the TFs are secondary targets. This seems possible as our microarray data shows that various genes encoding TFs were down-regulated in *polV* and *rdd* mutant backgrounds relative to the wild-type. In such a context, epigenetic regulation may be viewed as a secondary level of control superimposed on the primary level represented by transcription factors (Alvarez-Venegas et al., 2007). This cascade effect of regulation of genes via modulating the expression of TFs could also provoke a rapid increase of transcription of all genes within the network without the need to modify (prepare) each gene
individually. Such a control, perhaps, provides flexibility and rapid gene responses when required by the cell (Alvarez-Venegas et al., 2007).

A collaborative work carried out in Weixing Shan’s lab in China suggested that polV and rdd mutants were also susceptible to infection by Phytophthora, an important plant pathogen of oomycetes (Shan, unpublished). However, rdd plants infected with the necrotrophic pathogen B. cinerea did not show any susceptible phenotype relative to the wild-type plants (Kemal Kazan, personal communication). This indicates that the role of specific methylation and demethylation factors in plant defense may be evolutionarily conserved against type of pathogen.

I have also shown that in addition to PolV, another downstream component of the RdDM pathway, AGO4, is important for plant defense against F. oxysporum. Overall, our results highlight the importance of two antagonistic mechanisms, DNA methylation and demethylation in the regulation of plant immunity against F. oxysporum.

Future work can be devoted to comparative analysis of epigenomes and transcriptomes of polV and rdd, mutants during stress responses involving F. oxysporum infection. Chromatin immunoprecipitation technologies can be used to identify targeted genes. DNA methylation analysis of promoters of candidate genes identified by microarray techniques in susceptible mutants and wild-type plants before and after pathogen infection will shed more light on the functions and targets of DNA methylation, demethylation and histone modification in plant defense. In addition, proteomic analyses need to be carried out to identify potential protein partners and ultimately reconstitute regulatory complexes (Alvarez et al., 2010). Understanding such regulatory network would be an essential step towards development of potential tools for further exploitation towards sustainable agriculture.
REFERENCES.


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Diaz-Pendon, J. A., F. Li, et al. (2007). "Suppression of antiviral silencing by cucumber mosaic virus 2b protein in Arabidopsis is associated with drastically reduced accumulation of


Jones, A. L., C. L. Thomas, et al. (1998). "De novo methylation and co-suppression induced by a
Jones, L., A. J. Hamilton, et al. (1999). "RNA-DNA interactions and DNA methylation in post-
Nicotiana benthamiana demonstrates that extensive systemic silencing requires
Argonaute1-like and Argonaute4-like genes." Plant Physiology 141(2): 598-606.
Jost, J. P. (1993). "Nuclear extracts of chicken embryos promote an active demethylation of
glycosylase targets DNA demethylation." 25: 4545.
box protein p68 is tightly associated with the highly purified protein-RNA complex of 5-
MeC-DNA glycosylase." Nucleic Acids Res. 27: 3245.
embryosâ€”purification and properties of a 5-methylcytosine-DNA glycosylase." J. Biol.
Chem. 270: 9734.
DNA demethylation reaction in chicken embryo and G8 mouse myoblasts." FEBS Lett.
449: 251.
Arabidopsis life cycle is essential for parental imprinting." Plant Cell 18: 1360.
Mutants." Genetics 163(3): 1109-1122.
Kanno, T., B. Huettel, et al. (2005). "Atypical RNA polymerase subunits required for RNA-


Park, W., J. Li, et al. (2002). "CARPEL FACTORY, a Dicer Homolog, and HEN1, a Novel Protein, Act in microRNA Metabolism in Arabidopsis thaliana." Current Biology 12(17): 1484-1495.


cell 6(4): 791-802.


Plant Science 11(9): 460-468.


Wu, K., L. Zhang, et al. (2008). "HDA6 is required for jasmonate response, senescence and


**Plant Growth Media**

**MS media**

MS media is a high nutrient media used for general plant growth on plates under sterile conditions, which is made by preparing the following stock solutions:

### Macro (20x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>NH$_4$NO$_3$</td>
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</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>8.8 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
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</tr>
<tr>
<td>KNO$_3$</td>
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</tr>
<tr>
<td>KH$_2$PO$_4$</td>
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</tr>
<tr>
<td>dH$_2$O</td>
<td>to 1 L</td>
</tr>
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</table>

### Micro (1000x)

<table>
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</tr>
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<td>MnSO$_4$.4H$_2$O</td>
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</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
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<tr>
<td>H$_3$BO$_3$</td>
<td>3.11 g</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>4.3 g</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
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</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
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</tr>
<tr>
<td>KI</td>
<td>0.115 g</td>
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### Fe.EDTA (200x)

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<tr>
<td>FeCl$_3$.6H$_2$O</td>
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### Vitamins (100x)

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<tbody>
<tr>
<td>Nicotinic acid</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>20 mg</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>to 100 mL</td>
</tr>
</tbody>
</table>
To make 1 L of MS media:

<table>
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</thead>
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<tr>
<td>Macro</td>
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</tr>
<tr>
<td>Micro</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Fe.EDTA</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Vitamins</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 g</td>
</tr>
<tr>
<td>Myoinositol</td>
<td>0.1 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

Combine ingredients, adjust to pH 5.7 using 1N KOH, add 4.0 g Difco™ Agar (Bacto Laboratories) to each 500 mL and autoclave prior to use. For *F. oxysporum* inoculation assay on agar plates MS media without sucrose was used.

**MSN media**

MSN media has the same composition as MS media; with only half the concentration of NH₄NO₃ (16.5 g NH₄NO₃ is used to prepare the Macro (20x) solution).
APPENDIX II: Supplementary Methods

Purification of DNase-treated total RNA using the RNeasy® Mini Kit

DNase-treated total RNA was purified using the RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. Briefly, 700 μL RLT buffer containing 7 μL β-mercaptoethanol was added to 200 μL of prepared DNase-treated RNA. After vortexing, 500 μL of 100% ethanol was added, the solution was vortexed, and the mixture was transferred in two aliquots to an RNeasy Mini spin column, centrifuged for 20 sec at 10 000 g and the flow-through was discarded. The spin column was transferred to a new collection tube, 500 μL Buffer RPE was added, centrifuged at 10 000 g for 20 sec and the flow-through was discarded. The RPE buffer wash step was repeated, then the spin column was dried completely by spinning at 10 000 g for two minutes. DNase-treated RNA was eluted by adding 25 μL RNase-free dH₂O to the centre of the column, incubating at room temperature for one min, followed by centrifugation at 10 000 g for one minute. This step was repeated using a second aliquot of 25 μL RNase-free dH₂O, so that a total of 50 μL DNase-treated RNA was obtained.

Plasmid DNA minprep protocols

Qiagen QIAprep® Spin Miniprep Kit

Cells from 5 mL overnight E. coli suspension cultures were pelleted by centrifugation at 16 000 g for 1 min, then the pellet was resuspended in 250 μL P1 Solution and lysed in 250 μL P2 Solution. The mixture was incubated at room temperature for 5 min, 350 μL N3 Neutralisation Buffer was added, then the lysate was thoroughly mixed and centrifuged at 16 100 g for 10 min to remove cellular debris. The supernatant was added to the spin column provided in the kit to
allow the DNA to bind to the silica matrix, centrifuged at 16000 \textit{g} for 10 minutes, then the spin column was washed with 750 μL Buffer PE, centrifuged, and excess ethanol was removed from the column by centrifugation. The DNA was eluted with 50 μL Buffer EB, which was added to the column and incubated for 1 min prior to centrifuging to maximise DNA recovery.

**Alkaline/lysis method**

Cells from 5 mL overnight \textit{E. coli} suspension cultures were pelleted by centrifugation at 16 000 \textit{g} for 1 min, resuspended in 100 μL GTE buffer and then lysed by adding 200 μL of a freshly prepared 1:1 (v/v) solution of 2% SDS and 0.4 N NaOH. The lysate was incubated on ice for five minutes, 150 μL of 1.875 M NaOAc was added to neutralise the lysate, and the mixture was immediately centrifuged at 16 100 \textit{g} for 10 min to pellet cellular debris. The supernatant was transferred to a fresh tube, 400 μL chloroform was added, then the solution was mixed, centrifuged for one minute and the upper aqueous layer was transferred to a fresh tube. The DNA was precipitated by the addition of 0.7 volumes of 100% isopropanol, then the solution was thoroughly mixed and centrifuged at 16 100 \textit{g} for 10 min at room temperature. The pellet was washed with 500 μL chilled 70% ethanol, all traces of ethanol were removed using a pipette, then the pellet was air-dried for 10 min and resuspended in the desired volume of TE buffer containing 20 μg/mL RNase A.
Extraction of PCR products from agarose gels using the Qiagen QIAquick® Gel Extraction Kit

PCR products were extracted from agarose gels using the Qiagen QIAquick® Gel Extraction Kit, following the manufacturer’s instructions. The volume of excised agarose gel was estimated by weighing (1 mg is approximately 100 μL), then three volumes of Buffer QG were added and incubated at 50°C. Once the gel had completely melted, one volume of 100% isopropanol was added, mixed and the solution was passed through the provided spin column by centrifugation 14000 g for 2 minutes, then the column was washed using 500 μL Buffer QG. The column was washed with 750 μL Buffer PE containing ethanol, and the column was then centrifuged remove all traces of ethanol. The DNA was eluted using 30 μL Elution Buffer, which was added to the column and incubated for 1 min prior to centrifuging to maximise DNA recovery.

Phenol/chloroform DNA extraction

The same volume of phenol/chloroform/isoamyl alcohol (25:24:1; pH 6.6) was added to the grounded plant tissue, mixed by inversion, centrifuged for one minute at 16 100 g and the upper aqueous layer was transferred to a fresh tube. The same volume of chloroform as the original sample was added, mixed well by inversion, centrifuged at 16 100 g for 10 minutes and the aqueous layer was transferred to a fresh tube. A one-tenth volume (of the original sample) of 3M NaOAc and 2.5 volumes of 100% ethanol were added, the samples were mixed by inversion, incubated at -80°C for 10 min, then the samples were centrifuged at 15 700 g for 10 minutes at room temperature and the supernatant was discarded. The pellet was washed in 200 μL chilled 70% ethanol, centrifuged at 16 100 g for five minutes, all traces of ethanol were removed using a pipette, then the pellet was air-dried for 10 min and resuspended in 20 μL dH₂O.
APPENDIX III: Primers used in the study

<table>
<thead>
<tr>
<th>Primers used in this study</th>
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</tr>
<tr>
<td>CTTATCGGATTTTCTATGTTTGCC</td>
</tr>
<tr>
<td>iASKR</td>
</tr>
<tr>
<td>GAGCTCCTGTTTATTAACTTTGCTACATT</td>
</tr>
<tr>
<td>CutinaseF</td>
</tr>
<tr>
<td>TGGCGTCATCTTCATCTACG</td>
</tr>
<tr>
<td>CutinaseR</td>
</tr>
<tr>
<td>ACACCGTTTCTTGCCGCTACATT</td>
</tr>
<tr>
<td>F. oxysporum</td>
</tr>
<tr>
<td>GPDF</td>
</tr>
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</tr>
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<td>GPDR</td>
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<td>at1g23240R</td>
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APPENDIX IV: List of selected genes that were highly upregulated
or downregulated in the *polV* mutant

### Upregulated

<table>
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<th>SYMBOLS</th>
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</tr>
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<td>AT2G09187_1</td>
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<td></td>
<td>transposable element gene</td>
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<tr>
<td>AT5G41830_1</td>
<td>5.15</td>
<td></td>
<td>F-box family protein-related</td>
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<tr>
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<td>SDC</td>
<td>SDC (SUPPRESSOR OF DRM1 DRM2 CMT3)</td>
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<td>Encodes a defensin-like (DEFL) family protein.</td>
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APPENDIX V: List of selected genes that were highly upregulated or downregulated in the *rdd* mutant

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**Downregulated**

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