Gene silencing mechanisms in *Phytophthora infestans*

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Abstract

The hemibiotrophic oomycete, *Phytophthora infestans*, causes late blight on potato and tomato. This destructive pathogen is well known for the rapidity with which it overcomes host resistance. This thesis focuses on the molecular basis of gene (RNA) silencing in *P. infestans* and the role it may have in its genome biology and pathogenesis. A comparative genomic approach identified 51 genes from nine gene families, encoding Dicer-like (PiDcl1), Argonaute (PiAgo), RNA-dependent RNA polymerase (PiRdr1), Histone deacetylases, histone methyltransferases, DEAD helicases, chromodomain proteins, and a class 1 RNaseIII with a possible role in gene silencing. It was shown by knockdown of *PiDcl1*, *PiAgo1*, and *PiHda1* (Histone deacetylase) expression, that these components were involved in maintaining transcriptional (heterochromatic) silencing in *P. infestans*.

The large genome of *P. infestans* (~ 240 Mb) is composed of approximately 75% transposons and repeats. The genome has a bimodal architecture, consisting of a highly conserved and tightly packed core genome that is interrupted by transposons and repeats. The genes encoding the RxLR and Crinkler (CRN) effector classes that are critical to plant infection are predominantly located in the repeat and transposon rich regions. Small RNAs (sRNA) of 40 nt, homologous to a non-autonomous short interspersed retrotransposable element *infSINEm* were found. A sense orientation transcriptional fusion of *infSINEm* to the *PiAvr3a* (RxLR) effector gene led to silencing of both the introduced fusion and endogenous homologous sequences. Hence, it was concluded that *P. infestans* likely uses RNA silencing to minimize the activity of transposons and might influence expression of effector encoding genes in close proximity.

Deep sequencing of sRNA from different life cycle stages of two *P. infestans* isolates, that differ in their disease causing abilities, revealed 21, 25/26, and 32 nt size classes of sRNAs that were predominantly derived from transposons. Effector gene-derived sRNAs were also present in both isolates, but exhibited marked differences in abundance, especially for CRN effectors. Knockdown of Dicer and Argonaute gene expression provided evidence that biogenesis of 21 nt sRNAs is Dicer-dependent, while accumulation of longer sRNAs was impacted by silencing of Argonaute genes. Additionally, six miRNA candidates, and sRNAs that mapped to mitochondrial, tRNA and genomic hotspots were identified. sRNAs and RNA silencing in *P. infestans* have features characteristic of both plants and animals.

Keywords: gene silencing, oomycetes, *Phytophthora infestans*, potato, small RNAs

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Dedication

To my uncle Subba Rajugaru, aunt Vardhanama and my Parents

"Do not believe in anything simply because you have heard it. Do not believe in anything simply because it is spoken and rumored by many. Do not believe in anything simply because it is found written in your religious books. Do not believe in anything merely on the authority of your teachers and elders. Do not believe in traditions because they have been handed down for many generations. But after observation and analysis, when you find that anything agrees with reason and is conducive to the good and benefit of one and all, then accept it and live up to it."

Buddha

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Vetukuri, R.R.**, Avrova, A.O., Grenville-Briggs, L.J., van West, P., Söderbom, F., Savenkov, E.I., Whisson, S.C. and Dixelius, C. (2011) Evidence for involvement of Dicer-like, Argonaute, and Histone Deacetylase proteins in gene silencing in *Phytophthora infestans*. *Mol. Plant Pathol.* 12:772-785.

II. **Vetukuri, R.R**., Zhendong, T., Avrova, A.O., Savenkov, E.I., Dixelius, C. and Whisson, S.C. (2011) Silencing of the *PiAvr3a* effector-encoding gene from *Phytophthora infestans* by transcriptional fusion to a short interspersed element. *Fungal Biol.* 115:1225-1233.

III. **Vetukuri, R.R.**, Åsman, A.K.M., Tellgren-Roth, C., Jahan, S.N., Reimegård, J., Fogelqvist, J., Savenkov, E., Söderbom, F., Avrova, A.O., Whisson, S.C. and Dixelius, C. (2012). Biogenesis of small RNAs homologous to effector-encoding genes and transposable elements in the oomycete, *Phytophthora infestans*. (Manuscript)

IV. **Vetukuri, R.R.**, Åsman, A.K.M., Jahan, S.N., Fogelqvist, J., Reimegård, J., Avrova, A.O., Whisson, S.C. and Dixelius, C. (2012) Deep sequencing reveals diverse classes of small RNAs derived from genomic hotspots, tRNA and the mitochondrial genome in *Phytophthora infestans*. (Manuscript)

Additional publications

Bos, J.I.B. et al. (2010) *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proc. Natl. Acad. Sci. USA*, 107:9909-9914.

Whisson, S.C., **Vetukuri, R.R.**, Avrova, A.O. and Dixelius, C. (2012) Can silencing of transposons contribute to variation in effector gene expression in *Phytophthora infestans*? *Mobile Genetic Elements*. http://dx.doi.org/10.4161/mge.20265

The contribution of Ramesh R. Vetukuri to the papers included in this thesis was as follows:

I Participated in the planning of the project, performed most of the q-RT-PCR experiments, knockdown of Cdc14, and transient RNAi assays. Participated in analyzing data and manuscript writing.

II Participated in design of the project along with main supervisor and collaborators. Performed small RNA experiments, generated silenced lines of *Avr3a* and *SINEm-Avr3a*, performed initial qPCR analysis. Took part in writing of the manuscript.

III Participated in design of the project. Performed and evaluated majority of the experiments; validation of small RNA sequencing data by Northerns, mutant's analysis, qPCR analysis, 5´ and 3´ analysis of small RNA. Participated in manuscript writing.

IV Participated in design of the project. Evaluated bioinformatics data from the analysis. Participated in manuscript writing.

Abbreviations

Ago	Argonaute
CC	coil-coil domain
cAMP	cyclic adenosine monophosphate
CRN	Crinkle and necrosis effector; Crinkler effector
dsRNA	double stranded RNA
ER	endoplasmatic reticulum
lncRNA	long non-coding RNA
LRR	leucine-rich-repeat
miRNA	microRNAs
mRNA	messenger RNA
NB	nucleotide-binding site
ncRNA	non-coding RNA
piRNA	PIWI interacting RNA
Rdr	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
rRNA	ribosomal RNA
RxLR	Effector protein containing Arginine-any amino acid-Leucine-Arginine motif
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
sRNA	small RNA
ssRNA	Single stranded RNA
TE	transposable element
TIR	Toll/interleukin-1 receptor
tRNA	transfer RNA
UTR	untranslated region

1 Introduction

Due to its rapid spread and devastating effects, *Phytophthora infestans* is considered to be one of the most important pathogens in agriculture worldwide. In order to avoid damage due to this plant disease, intensive fungicide spraying programs are implemented to prevent infections. The costs of all control efforts and lost potato (*Solanum tuberosum*) production are estimated at 4.8 bn Euro (Haverkort et al., 2008). In Sweden in some years, up to 2/3 of all fungicide use is to combat *P. infestans* (for details see www.sjv.se). This is a practice (sometimes sprays every 4th or 5th day during "epidemic" seasons) that raises serious environmental concerns. Moreover, the quality of ware potatoes and seed potatoes can be severely affected due to post-harvest *P. infestans* infections of tubers. Details on fungicides recommended in Europe can be found at www.euroblight.net. In a climate change perspective, Sweden is predicted to have a more moist, cool, rainy and humid environment (IPCC, 2007), a situation that perfectly suits *P. infestans* infection. Thus, potato growers will face even more difficult conditions in future. Another emerging feature is that *P. infestans* now is widening its plant host range. Besides tomato, *S. nigrum* (nightshade) and other species such as petunia are reported to become diseased (Becktell et al., 2006; Flier et al., 2003a; Nelson, 2008). In Sweden, *S. physalifolium* (nattskatta) for example has proven to be very susceptible and able to sustain production of *P. infestans* oospores (Andersson et al., 2003). *P. infestans* also cause huge problems for tomato crops (Nowicki et al., 2012).

This summary will give a brief overview of potato and the late blight pathogen, *P. infestans*, followed by oomycete genomics and an introduction to the non-coding small RNA world. Due to the wide topics discussed, the text is biased towards the most recent findings and many reviews are cited to cover broader topics and various model organisms.

1.1 The potato crop, past and future

The first recorded European contact with potato was in 1537 in the Magdalena valley, Colombia. In South-America potatoes have been cultivated from several thousand years BC (Spooner et al., 2005). In particular, the Aymara Indians developed numerous varieties on the Titicaca Plateau. The Spanish invaders became familiar with the crop and it was probably circa 1570 that a Spanish ship first introduced potatoes to Europe. From Spain, potatoes became widely spread in Europe. It is thought that Swedish soldiers taking part in the so-called 30-year war (1618-1648) brought tubers with them when they returned to Sweden. Professor Olof Rudbeck planted potatoes in Uppsala Botanical Garden in 1658, as cited in his *Catalogus plantarum*. However, it was Jonas Alströmer (1685-1761) who made potato an acceptable crop among Swedish farmers and consumers. During the 1800s, potato became popular because it had been shown by Eva de la Gardie (1724-1786), the first woman in the Royal Swedish Science Academy (KVA), that potato could be used for alcohol production, replacing cereals that were needed for bread and other food products. Today, many very old potato varieties are still popular, such as Bintje and King Edward, both introduced into Sweden during the 1940s. Notably, Bintje was already on the Dutch market in 1910 (Erjefält, 1997), and King Edward on the British market in 1902 (Messer, 2000), and Russet Burbank in 1914 in the USA (Ortiz, 1998). The reason why consumers still prefer these and other old varieties are mainly for their cooking and flavor characters.

After wheat and rice, potato is the third most important food crop, with a worldwide production of 324 million tons in 2010 (faostat.fao.org). Optimization of production levels and resistance to biotic and abiotic stresses are key objectives of global potato breeding programs. Potato production is presently undergoing major changes. Until the early 1990s, most potatoes were grown and consumed in Europe, North America and countries of the former Soviet Union. Since then, there has been a dramatic increase in potato production and demand in Asia, Africa and Latin America, where the production increased from ~ 30 million tons per annum in the early 1960s to more than 192 million tons in 2010 (faostat.fao.org). In 2005, for the first time, the developing world's potato production exceeded that of the developed world. China is now the biggest potato producer, and almost a third of all potatoes are harvested in China and India. In 2010, the five largest producers were: China 74.7, India 36.5, Russia 21.1, Ukraine 18.7, and USA 18 million tons. The per capita consumption is highest in Europe, followed by North America and Asia. Potato has slowly become popular in Africa. In recent decades, production has increased from 2 million tons in 1960 to a record of 23.4 million tons in 2010. With this increase and geographic shift of production follows the problem with late blight. However, there is in general an absence of official data of yield losses worldwide incited by *P. infestans*. During epidemic years, potato crop losses are commonly estimated to exceed 75% without any crop protection (Oerke, 2006; Gildemacher et al., 2009;

Haverkort et al., 2009; Bhat et al., 2010; Hu et al., 2011). Losses of tomato are estimated to be on the same levels (Nelson, 2008; Nowicki et al., 2012; Fisher et al., 2012).

1.2 Potato and breeding

The tuber-bearing *Solanum* species are one relatively small group of a very large genus. Classical systematics classified those *Solanum* species in the subgenus Pachystemomum, section Tuberarium, subsection Hyperbasarthrum (Corell, 1962). The taxonomy of *Solanum*. species is confusing due to interspecific hybridizations that have occurred on several ploidy levels, a mixture of reproduction systems, and rather homogenous morphologies (Ovchinnikova et al., 2011) that mask the underlying speciation events. Hawkes (1990) divided the section *Petota,* to which *S. tuberosum* presently belongs, into 21 taxonomic series comprising 217 wild and seven cultivated species. A revision resulted in four cultivated and 100 wild species (Rodrígues et al., 2009; Rodrígues and Spooner, 2009). Further analysis on the origin of *S. tuberosum* suggests that hybridization events have occurred between closely related species, forming the cultivated species of today (Rodrígues et al., 2010).

Potato is a cool season crop. The optimum temperature for shoot growth and development is 22^{o}C. In spring, a soil temperature of about 24^{o}C is ideal whereas 18^{o}C is desired for good tuberization. The best potato production occurs in regions with daily growing season temperatures between 15 ^{o}C and 18^{o}C. The impacts of day length on flowering and tuberization are reviewed by Rodrígues-Falcón et al. (2006).

Fewer than ten related species have so far contributed to potato improvement by breeding. *Solanum tuberosum* is an autotetraploid species with 2n=4x=48 chromosomes. The basic chromosome number is x =12. Among the wild relatives a range of ploidy levels is present (2n = 24 or 48 alternatively 72), and few of the diploids 2n=24 have been explored in breeding. A biological feature of the wild potatoes is the contrast between very variable, outbred diploids and the less variable inbred polyploids, the former having a gametophytic incompatibility system and are highly intolerant of inbreeding. The polyploids are self-compatible and generally self-pollinated.

The modern potatoes, which emerged around the end of the nineteenth century, were all mainly genetically founded upon the original Andigena introductions (Hawkes, 1957). Wild species had no part in potato breeding until the early 1900s. Trait introgression from related *Solanum* species was intensified by the serious disease problems caused by potato late blight and nematodes. In the late 1960s, still only four varieties out of 30 harbored any genetic materials from a related species (*S. demissum*), and the most popular varieties were all pure *S. tuberosum* (Simmonds, 1976). Interestingly, non-tuber bearing *Solanum* species were used later, for example, to introduce virus resistance (Hermsen, 1984). Modern cultivars have been selected as single

successful recombinants from a large population of segregating progeny, and reproduced via vegetative propagation. Favorable recombinants are rare, and conventional potato breeding is time-consuming and expensive. Parental materials are heterozygous for most economically important characters, and large progenies have to be evaluated. Inbreeding depression resulting from selfing is usually severe in potato (Nakamura and Hosaka, 2010).

The major challenges in potato breeding are to develop new cultivars that give high yields but at the same time use water and nutrients more efficiently, meet consumer demands on flavor and health benefits, and harbor resistances to a range of insects (e.g. the Colorado beetle), nematodes and pathogens including *P. infestans*. To meet these demands, germplasm resources are important. Major collections and repositories include the International Potato Centre (CIP) in Peru, and the Commonwealth Potato Collection at the James Hutton Institute in Scotland, UK. For more information, see www.cipotato.org; www.hutton.ac.uk.

A new milestone in potato research came when the draft genome sequence was released (The Potato Genome Sequencing Consortium, 2011). The sequences derive from a double haploid genotype of the *Solanum phureja* group and a diploid breeding line from *S. tuberosum*, together constituting 844 Mb. In total, 39,031 protein-coding genes were annotated, and among those, 2,642 genes were clade-specific.

1.3 Resistance genes to plant pathogens

Plants have evolved two main strategies to detect pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). A plant host may have external pattern recognition receptor proteins (PRRs) recognizing conserved pathogen-associated molecular patterns (PAMPs). Activation of PRRs leads to PAMP-triggered immunity (PTI). The second layer of defense involves recognition by intracellular receptors (resistance proteins) of pathogen effector molecules leading to effector-triggered immunity (ETI). The co-evolving interactions between pathogen effectors and modified plant defense molecules over time form the basis of the so-called zig-zag model (Jones and Dangl, 2006). A model that have been revised several times, and modified also to better suit interactions to oomycete pathogens (Hein et al., 2009).

Resistance (*R*) genes in plants encode a few conserved and characteristic protein domains. These are typically a nucleotide-binding site (NB), leucine-rich-repeat (LRR) domains, and either a Toll/interleukin-1 receptor (TIR), or a coil-coil (CC) domain at the N-terminus. NB-LRR encoding genes comprise one of the largest gene families in plants. In an attempt to trace the origin of NB-LRR encoding genes, an extensive phylogenomic survey of 38 model organisms has been performed (Yue et al., 2012). The data showed that the NB-LRR domains existed separately before the split between prokaryotes and eukaryotes, and became fused together in land plants only; TIR domains seemed to have had an earlier origin.

Approximately 150 NB-LRR encoding genes have been identified in the genome of *Arabidopsis thaliana* (Meyers et al., 2003), 185 within *Arabidopsis lyrata* (Guo et al., 2011), 92 within *Brassica rapa* (Mun et al., 2009), 416 and 535 in the genomes of the woody species poplar and grapevine, respectively (Yang et al., 2008), 464 and 483 in two genomes of *Oryza sativa* (Yang et al., 2006), and 211 in the sorghum genome (Paterson et al., 2009). In addition, partial NB-LRRs that lack some NB-LRR specific domains and contain, for example, only TIR, TIR-NB, CC, and CC-NB domains, have been described in plant genomes (Meyers et al., 2002; Staal et al., 2008; Guo et al., 2011). The composition of NB-LRR genes varies significantly between species, exemplified by the low frequency of TIR-NB-LRRs in grass species (Tarr et al., 2009). Within genomes, NB-LRR genes are organized either as isolated single genes, or as linked clusters of varying size that are thought to facilitate rapid *R* gene evolution (Hulbert et al., 2001; McDowell and Simon, 2006). NB-LRR gene clusters are termed homogeneous when they contain only sequences that share a recent common ancestor. In contrast, clusters that contain more distantly-related NB-LRRs are referred to as heterogeneous (Friedman and Baker, 2007). When the annotated potato genome was assessed for NB-LRR gene types, 438 were found in the genome (Jupe et al., 2012). Of these, 57 harbor a TIR domain and 107 contained a CC domain. Physical map positions were also established for 370 of the 438 predicted NB-LRR genes across the 12 potato chromosomes. The majority of NB-LRRs are physically organized within 63 identified clusters, of which 50 are homogeneous in that they contain NB-LRRs derived from a recent common ancestor. Potato resistance gene sequences (TIR-NB-LRRs and CC-NB-LRRs) have earlier been compiled and genetically mapped to facilitate marker-associated breeding (Bakker et al., 2011). Moreover, the transcriptome of the sequenced *S. phureja* genotype, challenged with different abiotic stresses, elicitors and *P. infestans* is now publicly available (Massa et al., 2011). All this new information provides powerful resources for future improvements of the potato crop.

1.4 Late blight caused by *Phytophthora infestans*

Phytophthora literally means plant destroyer, a name given in 1861 by Anton de Bary, when he proved that a microorganism, at that time classified as a fungus, was the causal agent of late blight of potato. This was the plant disease responsible for the Irish potato famine in the 1840s (Eriksson, 1916; Large, 1940). After updated phylogeny analysis, *Phytophthora* species now are classified as oomycetes (water molds), a group of organisms related to brown algae (Kamoun 2003; Beakes et al., 2012). Other important plant pathogenic oomycetes are *Bremia lactucae*, *Plasmopara viticola*, *Hyaloperenospora parasitica* (all downy mildews), *Albugo candida* (white rust) and *Pythium* species (Agrios, 2005). Oomycetes can also be pathogenic on animals, for example *Saprolegnia parasitica* on fish, *Aphanomyces astaci* on crayfish, and *Pythium insidiosum* on humans (Phillips et al., 2008).

Oomycetes are members of the kingdom Chromista, and belong to the lineage of biflagellate "heterokont" organisms, commonly referred to as "stramenopiles" (Burki et al., 2007; 2008; Beakes et al., 2012). Together with alveolate species groups, which include the apicomplexa, ciliates and dinoflagellates (Figure 1), they constitute the diverse Chromalveolate "super-kingdom". More recent work suggests this "super kingdom" may also encompass the Rhizaria, where plant pathogens like *Plasmodiophora* and *Spongospora* presently are placed. Clearly, these phylogenetic relationships are still very complex. Apicomplexa include genera such as *Plasmodium* (malaria) and *Toxoplasma* parasites (Harper et al., 2005), that exhibit several common pathogenicity mechanisms and biochemical pathways with oomycetes (Bhattacharjee et al., 2006). Filamentous oomycetes are further divided into two orders, Saprolegniales and Peronosporales. The order Personosporales contains *Phytophthora* species and a number of other very important plant-pathogenic genera, including the genus *Pythium*. The genus *Phytophthora*, comprises 10 clades, and over 100 species (Kroon et al., 2012).

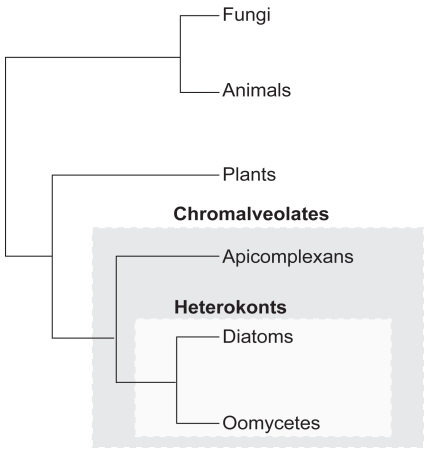


Figure 1. Simplified representation of the evolution tree of eukaryotic phylogeny showing selected groups of organisms (adapted from Gijzen, 2009). Oomycetes diverged from fungi > 600 million years ago. Branch lengths are not to scale.

The late blight pathogen originates from the central highlands of Mexico (Grünwald and Flier, 2005). The spread of *P. infestans* to the rest of the world, as infected potato tubers, occurred in two waves of migration. The first migration occurred in the 1840s and was represented by the A1 mating type. The next event happened in the mid-nineteen seventies when the A2 mating type was spread (*P. infestans* is heterothallic). The first report of the A2 mating type in Europe came in 1984 (Hohl and Iselin, 1984). This second step allowed sexual mating and recombination to occur which can potentially give rise to highly virulent strains (Figure 2).

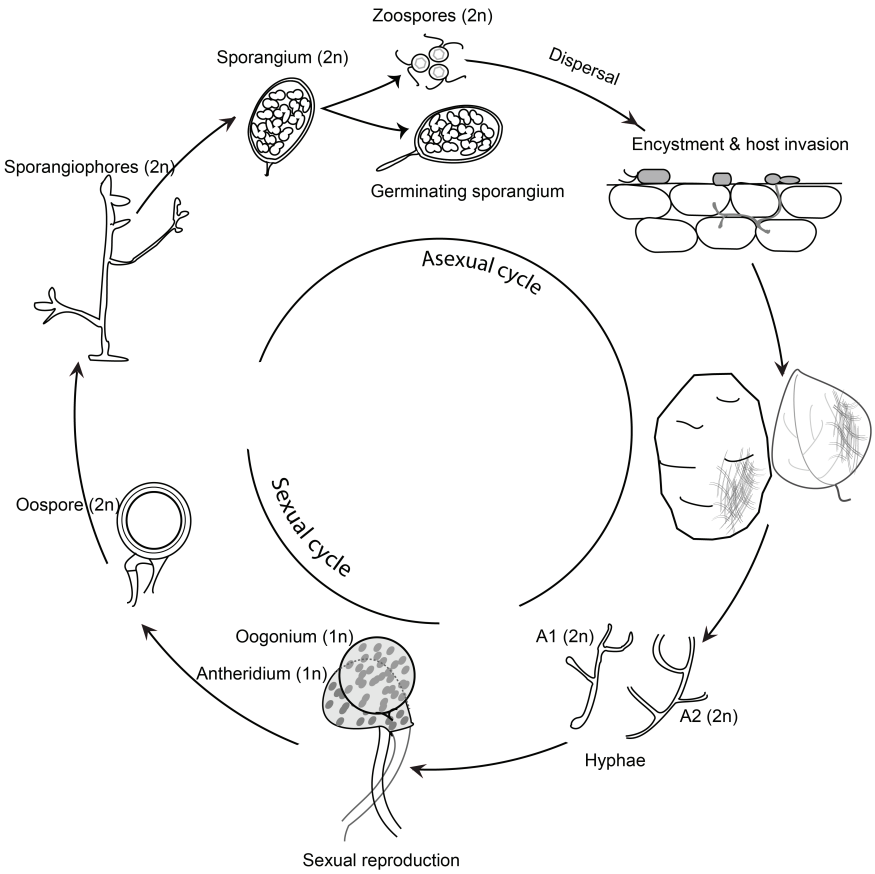


Figure 2. Life cycle of *P. infestans* Sporangiophores produce sporangia that can be blown long distances by wind. Sporangia produce zoospores that are only dispersed for a short distance. On contact with a host plant they germinate via the germ tube that forms and appressorium and penetrates in to the host. The mycelium grows intercellularly, invading the host tissue, and intracellular haustoria are formed. The disease spreads through out, more sporangiophores are formed, causing more infection cycles. When the two mating types, A1 and A2 are present in close proximity, *P. infestans* can reproduce sexually, since fertilization can take place between oogonium (female organ) and antheridium (male organ) forming oospores. Oospores are known to withstand harsh conditions. Progeny of the A1 or A2 type develop from the germinated oospores, providing a new source of inoculum to initiate infections.

Epidemiological studies have shown that A2 types are spreading faster than A1 and this shift in population structure has been observed both in Europe, North East Asia and North America (www.euroblight.org). These new populations also have greater capacity for spore production with the result that more sporangia (asexual spores) are produced per unit area of infected leaf. Another new feature is the shorter interval between infection and the appearance of

symptoms (the so-called latent period). The latter implies a need for shorter intervals between fungicide sprayings to prevent infections. The co-existence of the two mating types enables oospore formation and thereby soil infection (Andersson et al., 1998) making the pathogen even more difficult to control (Figure 3). Oospores can survive in frozen soil and form a germination peak that coincides with the potato planting in spring (Widmark et al., 2007). Thus, the disease potential of *P. infestans* is very high due to presence of both asexual and sexual stages, its soil borne inoculum (oospores, volunteers), and further long-distance spread via infected tubers or wind-borne sporangia. In Europe, these features have contributed to either a clonal type of *P. infestans* populations (Flier et al., 2007), or more genetically diverse as found in northern Europe where both A1 and A2 mating types are prevalent (Brurberg et al., 2011). Present control measures are either fungicides, or introduction of resistance genes in potato.

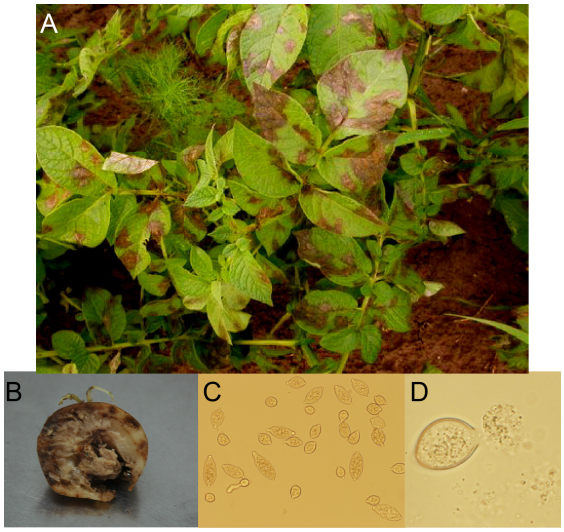


Figure 3. (**A**) *P. infestans* infection on potato leaves, and (**B**) tubers. (**C**) Sporangia, and **(D)** sporangia releasing zoospores.

1.5 Breeding for disease resistance

The potato crop, and also tomato crops, are frequently sprayed with fungicides (up to 10-15 times per season) to prevent disease from occurring. To minimize such chemical inputs and the devastating crop losses if no control measures are taken, resistance breeding has been prioritized for a long time. However, *P. infestans* has an extraordinary capacity to overcome all resistance (*R*) genes that hitherto have been introduced by the plant breeders (Fry, 2008). Several of first *R*-genes were introduced from wild species (e.g. *S. demissum*) but new *P.infestans* races that originated in the mid-1900s overcame these genes rapidly after market introduction (Turkensteen, 1993). Due to this backlash, breeders switched to introducing partial or quantitative resistance which was assumed to be of polygenic nature and much more durable. However, stacking these genes has proven to be very difficult due to the genetic out-breeding nature of potato. These types of traits are also strongly linked to late maturity, especially under long day conditions (Howard et al., 1970), and disappointingly also were broken by the pathogen (Flier et al., 2003b). Interest has again returned to wild *Solanum* species and new resistance resources found in *S. berthaultii, S. bulbocastanum, S. pinnatisectum, S. microdontum. S paucissectum, S. phureja* and *S. venturii* (Pel et al., 2009). In this context, the laborious work on the Toluca variety is of interest. It has taken the breeders 46 years to produce this variety from the wide cross between *S. tuberosum* and *S. bulbocastanum* in order to transfer resistance genes against *P. infestans* (*Rpi-blb2*) to *S. tuberosum* (Vleeshouwers et al., 2011). Unfortunately, many unwanted agronomical traits are still present in the Tuluca variety.

The present strategy today is not only based on introgression breeding as before, but is now accompanied by marker-assisted selection. However, cloning of *R*-genes, followed by stable transformation into existing highly productive potato cultivars is by far the fastest means of exploiting durable late-blight resistance in the *Solanum* gene pool. BASF Plant Science is the first company that is now entering commercialization of such genetically modified (GM) potatoes. They have cloned the *Rpi-blb1* and *Rpi-blb2*-genes from *S. bulbocastanum* (van der Vossen et al., 2003; 2005) and introduced them into the new cultivar Fortuna. Since BASF have abandoned the European GM-market in early 2012, the various genetic resources will now be introduced into North American and Asian potato cultivars; that is, genotypes adapted to other growing environments (Dixelius et al., 2012). This type of crop improvement may however be of less market value at the end if *P. infestans* starts to adapt to the *Rpi-blb* genes during the long GM related legislation processes.

1.6 Molecular tools for *P. infestans*

The strong interest from the agro-industry and academia to find ways to solve the problems with *P. infestans*, recent research breakthroughs in understanding *P. infestans* pathogenicity, and development of a range of molecular tools, has

led to an extremely intense research focus on this pathogen. Molecular biology tools for studying *P. infestans* include methods for transient and stable gene silencing (knock-down), reporter gene assays, and a wealth of genetic information that includes >80,000 expressed sequence tags (ESTs) and a sequenced genome (Haas et al., 2009). This has established *P. infestans* as a model oomycete organism for studying the pathogen´s molecular and biochemical processes underlying disease development (Judelson, 2007). The genome of *P. infestans* is much larger than those found in the closely related species *P. capsici* (65 Mbp; 12,011 genes), *P. ramorum* and *P. sojae* (Table 1). This difference is mainly due to larger amounts of repetitive DNA and transposons. It is estimated that approximately half of the *P. infestans* genome contains mobile elements plus 25% of additional repeats. The mobile elements are highly expressed and are associated with expansion of disease-promoting effector gene families and genome reorganizations (Haas et al., 2009). Recent years have witnessed a renaissance in molecular biology research into oomycetes, culminating in the genome sequencing of several plant pathogenic species, besides *Phytophthora,* and the discovery of vast numbers of effector proteins (Tyler et al., 2006; Baxter et al., 2010; Levesque et al., 2010; Kemen et al., 2011; Links et al., 2011).

1.7 Effectors

The effector concept in the area of plant-microbe interaction appeared more widely during the mid-2000s, mostly due to the discovery of the type III secretion system in *Pseudomonas syringae* and its role for effector delivery to plant cells. Today, “effector” has a rather broad definition and includes any secreted pathogen proteins and other molecules that modulate plant defense circuitry and enable parasitic colonization of plant tissue, including toxins, and enzymes (Kamoun, 2007; Hogenhout et al., 2009).

The genome of *P. infestans* codes for extremely large numbers (>700) of effector proteins (Haas et al., 2009; Raffaele et al., 2010) that target different sites in infected host plant tissue (Kamoun, 2006; Schornack et al., 2009). One effector group acts in the extracellular space (apoplast) where they interfere with apoplastic plant defenses. Inhibitors of plant extracellular proteases and glucanases are examples of such apoplastic effectors. Small cysteine-rich proteins (SCRs), are also thought to function in the apoplast but their effector activities remain mostly unknown. Interestingly, the cystatin-like effector proteins (EPICs) that inhibit secreted host proteases during infection most likely have co-evolved with plant proteases, mimicking an arms race between enzyme and inhibitor at the plant-pathogen interface (Kaschani and van der Hoorn, 2011). Six plant pathogenic oomycetes are sequenced to date, with various genome sizes, and a broad repertoire of effector proteins (Table 1).

Table 1. *Predicted genome information including selected effector classes in plant pathogenic oomycetes.*

Species	Genome size Mb	Gene Models	RxLR	CRN	CHxC	NLP[a]
Phytophthora infestans[1]	240	17,797	563	196	0	27
Phytophthora ramorum[1]	65	15,743	350-374	19	0	40-59
Phytophthora sojae[1]	95	19,027	350-396	100	0	29-37
Hyaloperonospora arabidopsidis[2]	99	14,543	134	20	0	10
Albugo candida[3]	45	15,824	26 RxL	6	40	0
Pythium ultimum[4]	43	25,290	91YxSL	26	0	7

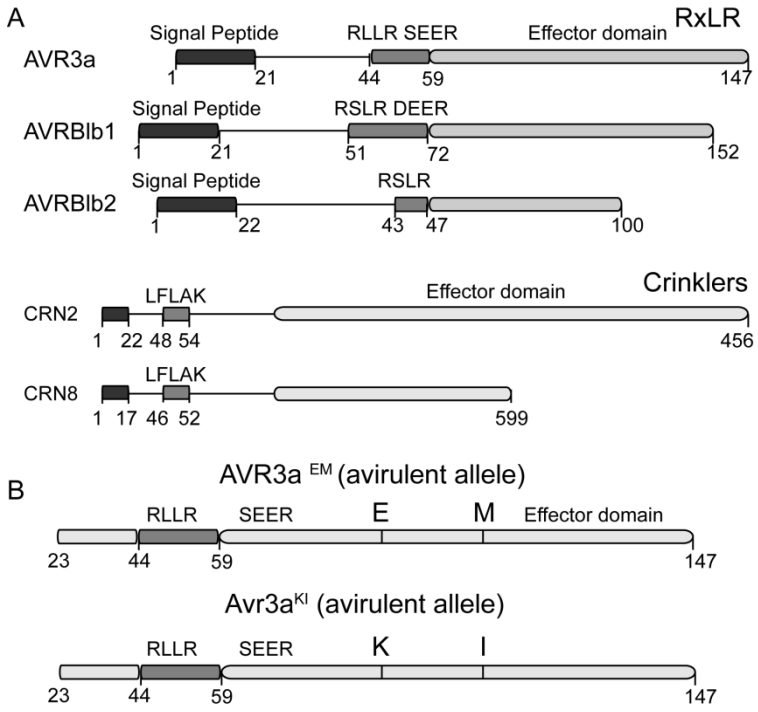
1= Haas et al., 2009; 2= Baxter et al., 2010; 3= Links et al., 2011; 4=Levesque et al., 2010.
a=necrosis and ethylene-inducing (NLP).

A large number of *P. infestans* effectors, classified as cytoplasmic effectors, are delivered inside host cells using N-terminal secretion and host-translocation signals (Figure 4). This is the case for members of the genes encoding secreted effectors with the RxLR amino acid motif (arginine, any amino acid, leucine, arginine) and Crinkle and Necrosis (CRN) families. CRNs contain a LxLFLAK motif, often followed by a conserved DWL domain at the N-terminal end, whereas the C-terminal domains show high diversity (Haas et al., 2009). A subset of the RxLR effectors is recognized inside plant cells by intracellular immune receptors of the NB-LRR resistance protein family, resulting in the induction of hypersensitive cell death and immunity (Whisson et al., 2007; Birch et al., 2008).

AVR3a is one of the most studied RxLR effector proteins from *P. infestans*. It has for example been shown that it manipulates plant immunity by stabilizing the plant ubiquitin E3-ligase CMPG1 that is required for cell death mediated by infestin 1 (inf1) elicitin (Bos et al., 2010). Recently, it was discovered that the PiAvrBlb2 RxLR effector protein enhances susceptibility of a host plant by targeting the host papain-like C14 cysteine protease, specifically preventing its secretion into the apoplast (Bozkurt et al., 2011). This also demonstrates how sophisticated the arms race between *P. infestans* and its host plants has become during their co-evolution. An emerging concept in antagonistic host-pathogen interactions is that effector targets are under diversifying selection to evade manipulation (Hogenhout et al., 2009). Chitinases and glucanases are good examples (Bishop et al., 2005), including the pathogen glucanase inhibitor GIP1 (Damasceno et al., 2008). Further, extensive evolutionary and comparative genomic analyses have shown that *Phytophthora* effector genes have undergone accelerated patterns of birth and death evolution, with evidence of extensive gene duplication and gene loss in the genomes of *P. infestans*, *P. sojae*, and *P. ramorum*, potentially explaining their capacity to adapt to a changing host environment (Win et al., 2007; Jiang

et al., 2008). Knowledge of effectors in *P. infestans* has lately been suggested as a tool for exploitation in future control measures (Vleeshouwers et al., 2011; Whisson et al., 2011).

Comparative analysis of fungi has revealed numerous gene acquisitions, gene duplications and gene losses among ascomycete fungi (Wapinski et al., 2007), and recently within the Chromalveolata (Kemen et al., 2011). Typically, secreted proteins from fungal plant pathogens have low sequence similarity to any known protein and there is little understanding of their functions (Koeck et al., 2011). A large number of fungal genomes are now publicly available (Soanes et al., 2008) and comparison of gene inventories of pathogenic and non-pathogenic organisms representing different subfamilies in fungi offers a direct means to provide new information on important PAMPs and effector molecules and their evolution. One interesting finding is the rather frequent events of horizontal gene transfer from fungi to oomycetes, explaining how the latter organisms may have become plant pathogens (Richards et al., 2011).



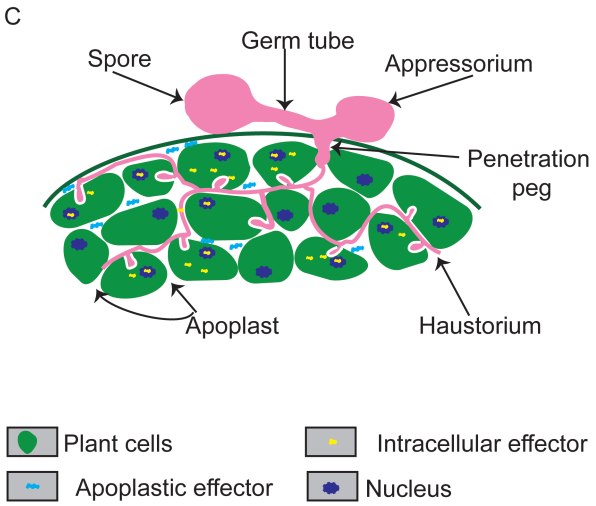


Figure 4. Schematic representation of cytoplasmic effectors in *P. infestans,* and their signature motifs (modified from Armstrong et al., 2005; Kamoun, 2007). (**A**) N-terminal targeting and C-terminal effector domain organization of RxLR and Crinklers (CRN): AVR3a, Avr1blb1, AVRblb2, CRN2 and CRN8. The numbers under the sequences indicate amino acid positions. Both effectors have a signal peptide region and signature motifs RxLR-(dEER) and LFLAK (CRN). (**B**) Schematic view of avirulent and virulent allelic forms of AVR3a, differing by two amino acids as indicated. (**C**) Model illustrating oomycete effector deployment during colonization. Oomycetes secrete apoplastic and cytoplasmic effectors to facilitate host colonization. Apoplastic effectors are secreted into the extracellular space where they interfere with plant defenses. Cytoplasmic effectors are translocated inside host cells and modulate host defense machinery. Not drawn to scale.

1.8 Gene silencing mechanisms and noncoding RNAs

The central dogma states that flow of information in biological systems occurs from DNA via RNA and to proteins. The role of RNA in the information flow is viewed as being more of a passive, intermediary nature. Three different types of RNA (messenger RNA, transfer RNA and ribosomal RNA; mRNA, tRNA, rRNA) convert the code from DNA into polypeptides. However, research in the past 50 years have shown that RNA does much more than simply play a role in protein synthesis. The roles that RNA molecules play are wide ranging from catalytic function to regulatory and information storage. Generally those RNA molecules that do not code for any proteins are termed non-coding RNAs (ncRNAs). These are functional entities that include both large and small RNAs (sRNA), and include the most abundant classes of RNAs such as rRNAs and tRNAs, and also sRNA classes. sRNAs come in different sizes and with different characteristics. They are present in sizes ranging from 16-40 nucleotides. To date, many classes of sRNAs have been described in numerous organisms across different kingdoms. They are classified based on their biogenesis, interacting protein complexes, functions and targets. miRNA,

exo-siRNA, endo-siRNA, snoRNA, scaRNA, piRNA and rasiRNA are some of the well-studied sRNAs (Ghildiyal and Zamore, 2009). New classes of sRNAs continue to be discovered with new sequencing technologies.

sRNAs are major players in RNA-mediated gene silencing mechanisms. Gene silencing, or RNA silencing, was accidently discovered in experiments attempting to make petunia petals purple by over-expressing chalcone synthase, which unexpectedly resulted in white flowers (Napoli et al., 1990). The transcript abundance of the endogenous gene, as well as the introduced transgene was 50-fold lower, compared to wild-type petunia petals. As the introduced copy led to co-suppression of the endogenous gene, the term co-suppression was coined. A similar phenomenon, termed quelling, was observed in the fungus *Neurospora crassa* (Romano et al., 1992; Sen and Blau, 2006). In 1998, six years after the discovery of co-suppression and quelling, Fire et al. (1998), working with the nematode *Caenorhabditis elegans,* showed that double-stranded RNA (dsRNA) was the silencing trigger in gene silencing (Fire et al., 1998), and provided an explanation for the previously reported silencing of endogenous genes.

Gene silencing or RNA silencing can be defined as sequence-specific regulation of endogenous and exogenous genes originating from different sources (Figure 5). RNA silencing is initiated by long dsRNA or hairpin-structured RNAs which are cleaved to produce 21-40 nt small double-stranded RNA duplexes (dsRNAs). The resulting dsRNAs are loaded into a Ribonucleoprotein (RNP) complex, also called the RNA-induced silencing complex (RISC). In the dsRNA duplex, the strand that directs silencing is “the guide strand”, whereas the other strand, which is degraded, is “the passenger strand”. The dsRNAs are separated into single stranded RNAs in association with RISC complex, and guide the complex to the homologous target transcript. This results either in transcript degradation or translational repression (Kawaji et al., 2008).

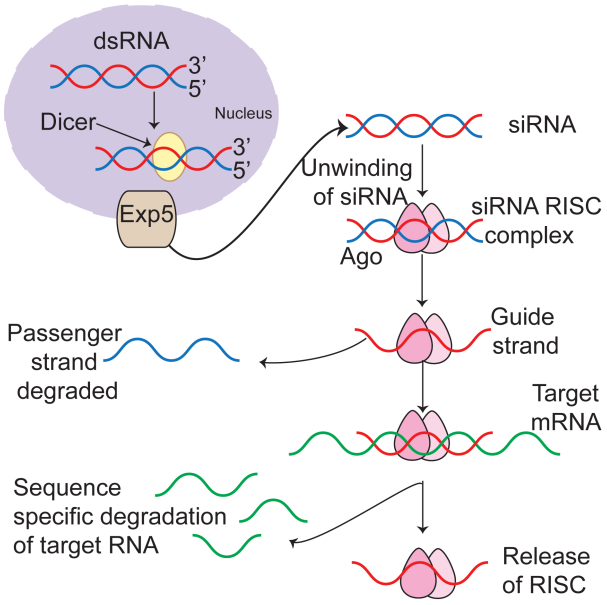


Figure 5. Simplified illustration of the RNA silencing pathway. Double-stranded RNA precursors of different kinds from exo- and enodogenous sources are processed by a Dicer protein into short duplexes (16-40nt) containing guide and passenger strands. These short duplexes are incorporated into the RNA-induced silencing complex (RISC) that comprises Argonaute and other proteins. In the duplex the passenger strand is separated and degraded, whereas the guide strand directs Ago/RISC to the target RNA. The target is recognized, its expression is modulated either by repression or sequence specific degradation, depending on the biological context.

1.8.1 The essential gear of the RNA silencing pathway

At the heart of RNA silencing pathways are two ribonucleases that take part in initiator and effector steps of silencing mechanisms. There is also a third player, Rdr which takes part in amplifying and maintaining the silencing signal in many organisms.

1.8.2 Dicer: the initiator

The name Dicer was given by Emily Bernstein who first demonstrated the enzyme's dicing capability (Bernstein et al., 2001). Dicer, along with Drosha proteins, belong to the RNase III family, they both take part in the initiator step of the RNA silencing pathway, generating small siRNA duplexes from long dsRNA or precursor microRNAs. The Dicer protein is a well-conserved family present across different kingdoms of life (de Jong et al. 2009). RNase III enzymes are classified into three classes (Figure 6) Class I enzymes are predominately found in bacteria, bacteriophages, and some fungi like yeast, and contain single RNase III domain and a dsRNA binding domain (dsRBD). Class I enzymes process precursor RNAs to ribosomal RNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA). Drosha, with two RNase

domains, is the classical example of class II enzymes, and takes part in the miRNA pathway. Class III includes Dicer family proteins which generally contain up to six types of domains including a DEAD box, helicase C-terminal, DUF283, Piwi-Argonaute-Zwille (PAZ), RNase III, and dsRBD (Hammond, 2005; Margis et al., 2006; Liu et al., 2005). Some of the Dicer or Dicer-like proteins from lower eukaryotes may lack one or more of these domains. For instance, the Dicer protein from *Giardia intestinalis* (a protozoan parasite) contains only a PAZ and two RNase III domains (MacRae et al., 2006). The helicase domain of Dicer functions by dissociating the RNA duplex in an energy dependent manner using ATP in *Drosophila*, whereas in *Homo sapiens* it is an ATP independent reaction (Ji, 2008). The PAZ domain is involved in substrate recognition and functions as a ruler for siRNA product size determination. A long α helix connects the PAZ domain to the RNase IIIa domain of Dicer. The DUF283 domain is proposed to be involved in siRNA/miRNA strand selection. Two RNase III domains, named as RNase IIIa and b, are strictly conserved in all RNase III proteins. The two RNase III domains form a catalytic dimer. This is an intermolecular homodimer for class I enzymes in prokaryotes, and an intramolecular pseudodimer between RNase IIIa and IIIb domains for Dicer and Drosha in eukaryotes. The cleavage of long dsRNA occurs when Dicer binds to an existing terminus of dsRNA and makes a 21 nt cut from the end. The RNase III dimers take part in dsRNA cleavage, with each RNase III domain cleaving one strand of the dsRNA, generating siRNAs with a 5´ phosphate and 2 nt 3´ overhangs (Hammond, 2005; Du et al., 2008).

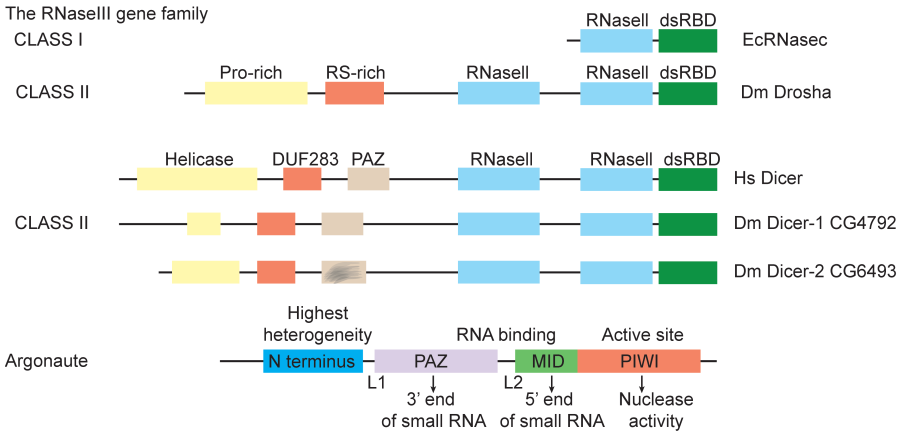


Figure 6. Domain organization of RNase III gene family. Three different classes of RNase III genes are shown with their signature domains. Abbreviations shown in the figure are: Ec: *E. coli*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens* (adapted from Hammond, 2005). Domain organization of Argonaute proteins (adapted from Ender and Meister 2010).

The Drosha enzyme takes part in miRNA pathways in animals. To date it has not been found in plants or other eukaryotes. This enzyme processes primary miRNAs, with the manner of enzyme-substrate binding differentiating it from Dicer. Drosha recognizes the stem-loop structure, the sequences flanking the stem loop, and releases the stem-loop from the primary miRNA. Following this step, the stem-loop is exported to the cytoplasm by Exportin-5 to be further processed by Dicer (Hammond, 2005).

There is wide variation in the number of Dicer proteins in plants, compared to animals and fungi, for example *A. thaliana* and *O. sativa* contain four and five Dicer proteins, respectively, whereas most metazoans are thought to contain either a single Dicer (*C. elegans*, vertebrates) or two Dicers (*Drosophila*) (de Jong et al., 2009). In fungi, the number of Dicers can range from one (*S. pombe*) to three (*Coprinopsis cinerea*) (Nunes et al., 2011b). The higher number of Dicers is attributed to diverse silencing pathways, and to their requirement in immune defense in plants.

1.8.3 Argonaute: the effector of silencing pathways

As the phenotype of AGO knockout in *A. thaliana* resembled tentacles of the octopus *Argonauta argo*, the name Argonaute was given to the protein associated with the phenotype (Ender and Meister, 2010). Argonautes are well conserved and are expressed in bacteria, archaea and eukaryotes. However, their numbers vary greatly in different species. For instance, there is one Argonaute protein in *S. pombe*, 27 in *C. elegans*, eight in humans, five in *Drosophila* and ten proteins in *A. thaliana* (Hutvagner and Simard, 2008). Argonaute proteins are defined by four distinct domains (Figure 6), being the N terminal domain, the PAZ, Mid, and PIWI domains. The N terminal domain is a variable region that aids in separation of sRNA from the target after slicing. The PAZ domain is common to Dicer and Argonaute proteins, consisting of two subdomains. The PAZ domain can recognize the 3′ ends of sRNA duplex, due to their 3′ overhangs; this feature helps it to differentiate regulatory sRNAs from degraded RNAs. Crystallographic studies on Argonaute proteins from the bacterium *Thermus thermophiles*, and the archaeon *Archaeoglobus fulgidus*, have revealed that the PIWI domain resembles an RNase H domain-containing protein from *Bacillus holodurans*. RNase H proteins cleave the RNA strand of an RNA-DNA hybrid using DNA as template (Jinek and Doudna, 2009) and their catalysis requires a conserved Asp-Asp-Glu/Asp motif in the catalytic core. The signature cleavage products of PIWI contain 3′ OH and 5′ monophosphates (Hutvagner and Simard, 2008; Mallory and Vaucheret, 2010).

Argonaute proteins are divided into two subgroups based on sequence homology. Argonautes which resemble *A. thaliana* AGO1 belong to the Ago subfamily, whereas those that resemble *Drosophila* PIWI proteins are referred to as the Piwi subfamily (Ender and Meister, 2010). Piwi subfamilies are restricted to animals, and are active especially in germline cells, where they associate with piRNA involved in genome defense against transposons (Siomi et al., 2011). As the first studied Argonaute protein catalyzed targeted mRNA

cleavage, the protein was termed the 'Slicer' protein. However, this term cannot be applied to all Argonautes, as some act only by repressing target gene expression (Mallory and Vaucheret, 2010). Translation repression occurs when complementarity beyond the seed region (2 to 7 nt) is imperfect; this occurs mostly in animals. In addition to post-transcriptional gene silencing, Argonaute-mediated cleavage can be linked to DNA methylation and heterochromatin formation. Whether the Argonautes act by repressing the target expression or target cleavage depends on the complementarity between the small RNA and its target.

The factors that influence the sorting of sRNAs into different RISCs containing various Argonautes differ in plants and animals. In animals, sorting is influenced by sRNA duplex structure and to some extent on terminal nucleotide, whereas in plants it is the 5′ terminal nucleotide and s RNA length that contribute to sorting of sRNAs. For example, sRNAs of 21-22 nt with U at the 5′ terminal ends are sorted into AGO1 in *A. thaliana*, whereas sRNAs with a 5′ terminal A are associated with the AGO2 complex (Czech and Hannon, 2011). Apart from Dicer-dependent pathways, Argonautes are also involved in Dicer-independent silencing pathways (Siomi et al., 2011).

1.8.4 RNA dependent RNA polymerase (Rdr): Silencing amplifier

Rdr is defined by a conserved RNA-dependent RNA polymerase domain and some of the Rdr that take part in silencing pathways catalyze the replication of RNA from an RNA template. It can also synthesize dsRNA molecules using a single-stranded RNA template (ssRNA) molecule or siRNA duplex as a template in a primer independent or dependent manner (Figure 7). Rdr is present in plants, animals and fungi, but is lacking in *Drosophila*, mice, and humans. Rdr executes a variety of different cellular functions (Willman et al., 2011).

In many eukaryotes, the post-transcriptional silencing initiated by Dicer and executed by Argonaute proteins is enhanced by Rdr. The siRNA from the Argonaute complex that binds to the target mRNA trigger Rdr to generate primer dependent dsRNA that is eventually processed into secondary siRNA by Dicer dependent mechanisms (Crombach and Hogeweg, 2011). In *A. thaliana*, RDR2-generated siRNA aid DNA methylation and histone modifications at telomeres via RNA-directed DNA methylation (RdDM) (Willman et al., 2011). In *S. pombe*, Rdp1 (Rdr) is essential for centromeric silencing, heterochromatin assembly, chromosome segregation, and telomere clustering during mitosis (Sugiyama et al., 2005).

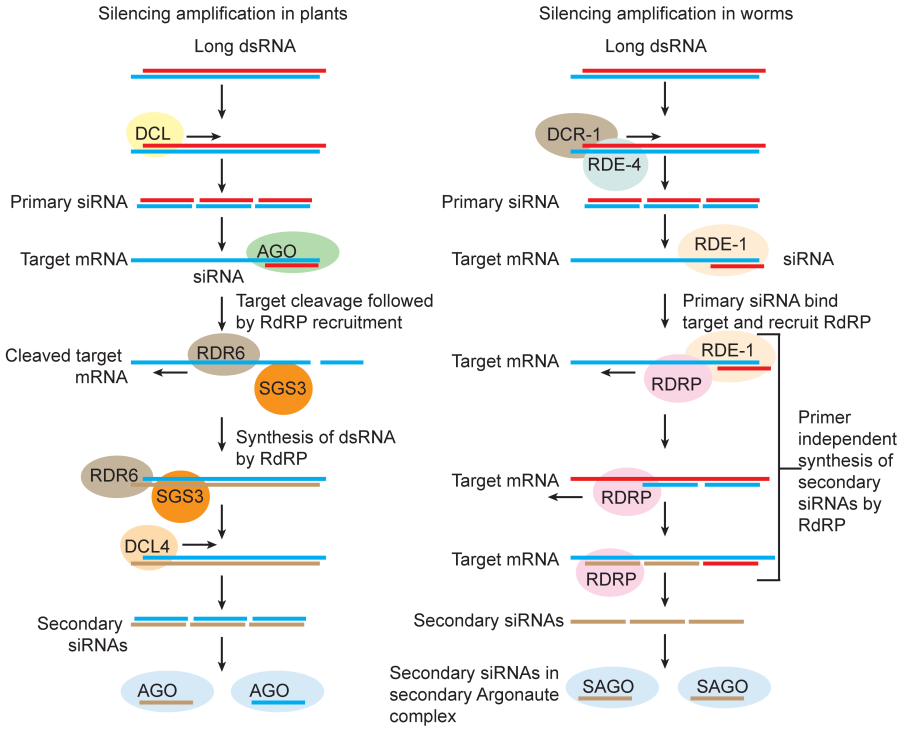


Figure 7. Schematic of RNA dependent RNA polymerase (Rdr) silencing in plants and worms. Rdr also known as RDR in *Arabidopsis*, amplify the silencing signal generated from exogenous dsRNA. RDR (RDR6) use cleaved target fragments as template to synthesize dsRNA, these dsRNA further enter the Dicer dependent silencing pathway and generate secondary siRNA. In *C. elegans* on the other hand primary siRNA generated from long dsRNA acts as guide to aid Rdr (RdRP) in amplifying the silencing signal (adapted from Ghildiyal and Zamorem, 2009).

In *C. elegans* there are four Rdr paralogs involved in efficient gene silencing. One of the four Rdrs, RRF-3, is proposed to mediate synthesis of secondary siRNAs. *C. elegans* secondary siRNAs are reported to be initiated via a primer independent mechanism (Sijen et al., 2001, Lee et al., 2006; Pak and Fire 2007). The end product of RRF-1 processing is single stranded siRNA bearing 5' triphosphates (Pak and Fire, 2007), which differ from Dicer processed siRNA that have 5' monophosphate.

1.8.5 Small RNAs (sRNAs)

Even though many new classes of sRNAs have been discovered over the years along with new distinct silencing pathways, these pathways are somehow interconnected and rely on each other for substrates and accessory proteins. One such example is the link between the tasiRNA and miRNA pathways in *A. thaliana*, where a miRNA mediated pathway directs cleavage of At2g27400

transcript into tasiRNAs (Vazquez et al., 2004). There are many more examples of such cross talk, reviewed in Ghildiyal and Zamore (2009). In spite of the discovery of new sRNA classes, only three main classes of sRNAs have been at the forefront of RNA biology research: siRNA, miRNA and piRNAs. In this thesis there will be greater focus on miRNA and siRNA. Both siRNA and miRNA are found in all the kingdoms of eukaryotic life and are characterized by the double-stranded nature of their RNA precursors. siRNA originate from exogenous and endogenous sources, whereas miRNA originate from endogenous transcripts that form stem-loop structures. Piwi interacting RNAs (piRNA) have only been found in animals to date. They appear to be derived from single stranded precursors and are involved in regulating transposable elements in the germline (Malone and Hannon, 2009).

1.8.6 siRNA

siRNA were initially discovered in plants in virus induced gene silencing experiments (Carthew and Sontheimer, 2009). There are a number of subclasses of siRNA that are derived from different pathways, are of different sizes, and rely on distinct proteins to exert their function. They are the first line of defense against invading viruses and transposons and aberrant transcription products. They are generated via Dicer-dependent or Dicer-independent pathways and all originate from long dsRNA. The silencing signals generated via Dicer pathways are amplified by RNA-dependent RNA polymerase (Rdr) in the presence of cleaved primary transcripts, using them as template to generate long dsRNA to prime further silencing (Ghildiyal and Zamore, 2009). Based on the progenitor source (dsRNA) for siRNAs, they are classified as exo-siRNAs and endo-siRNAs. In animals exogenous dsRNA originate from transgene or viral RNAs. However, in plants, apart from virus, progenitors for exogenous siRNA may originate from single stranded sense transcripts of transgenes that are converted to dsRNA by RDR6, belonging to the RNA-dependent RNA polymerase family (Qu et al., 2005).

To date, endo-siRNAs have been detected in a wide variety of organisms such as plants, *C. elegans*, *Saccharomyces castellii*, *Candida albicans*, *Magnaporthe oryzae*, *Drosophila* and mouse oocytes (Nilsen, 2008; Kritikou, 2008; Drinnenberg et al., 2009; Nunes et al., 2011a). Endo-siRNAs are produced by the action of Rdr. However in organisms lacking Rdr, such as *Drosophila* and mouse to name a few, endo-siRNAs are derived from mobile genetic elements, long hairpin structures generated by the transcription of palindromic sequences, overlapping RNAs formed by convergent transcription and cognate pseudogenes (Czech et al., 2008; Kawamura et al., 2008; Nilsen, 2008). In animals it is believed that endo-siRNAs have a role similar to piRNAs, which act by suppressing the expression of transposons (Kritikou, 2008). Studies on *Drosophila* showed that endo-siRNA have distinct features that include: 21nt size, Dicer-dependent origin, present in both sense- and antisense orientation, modified 3′ ends, and 5′ nucleotide not biased to uracil (Katiyar-Agarwal et al., 2006; Kawamura et al., 2008; Okamura et al., 2008;

Ghildiyal and Zamore, 2009). In *C. elegans*, transcript accumulation from transposons increases in both Dicer and Argonaute mutants, highlighting the role of endo-siRNA in control of transposons (Tabara et al., 1999; Ketting et al., 1999).

In plants there are many subclasses of endo-siRNAs such as (for example), cis-acting siRNAs (casiRNA), trans-acting siRNAs (tasiRNAs), and natural antisense transcript-derived siRNAs (natsiRNAs). Of the four Dicer (DCL), three Rdr and ten Argonaute (AGO) proteins having unique functions in *A. thaliana*, DCL3, RDR2 and AGO4 are associated with endogenous casiRNAs (Dalmay et al., 2000; Mourrain et al., 2000; Park et al., 2002; Reinhart et al., 2002, Schauer et al., 2002; Yu et al., 2003 Ziberman et al., 2003). In *dcl3* and *rdr2* mutants, loss of endogenous casiRNA was associated with loss of heterochromatic silencing and increased transcript accumulation (Xie et al., 2004). These siRNAs are approximately 24 nucleotides in size and methylated by HEN1, and arise from highly repeated sequences and transposons.

Another class of siRNA that is well described in *A. thaliana* is tasiRNA. As the name suggests, these siRNAs act in *trans*. They are 21nt siRNAs, corresponding to sense and antisense strands, and direct cleavage of mRNA. tasiRNAs are one of the unique class of sRNA that require components of both siRNA and miRNA pathways for biogenesis (Vazquez et al., 2004; Peragine et al., 2004). They arise from miRNA-directed cleavage of RNAs from *TAS* loci, yielding cleaved transcripts which are then converted to dsRNA by the action of RDR6, followed by DCL4 cleavage of dsRNA into phased tasiRNAs (Allen et al., 2005). In response to biotic and abiotic stress, *A. thaliana* produces natsiRNAs that are 21-24 nt in size (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). NatsiRNA are dependent on DCL1, 2, RDR6 and SG3 and Pol IV for their biogenesis. Bidirectional transcripts induced by stress are the source of dsRNA trigger for these siRNAs.

1.8.7 miRNAs: The master regulators

In a discovery that emerged as a giant leap in understanding of gene regulation, it was found that the *lin-4* gene, required for post-embryonic development in *C. elegans*, produced a 22 nt RNA partially complementary to the 3´UTR region of its target, *lin14* (Lee et al., 1993). This is the beginning of fascinating research about miRNA, In terms of evolutionary time scale, miRNAs are considered to be 400 million years old. They are often well conserved within plant and animal kingdoms (Millar and Waterhouse, 2005), but there are no reports of miRNAs that are conserved across all the kingdoms.

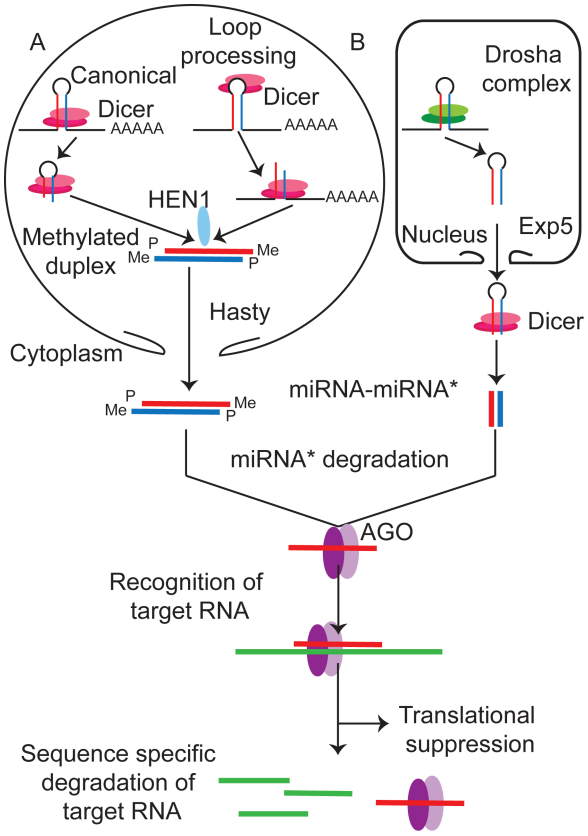


Figure 8. miRNA biogenesis pathway in plants and animals. (**A**) In plants, following transcription, the pri-miRNAs are processed by the nuclear RNase III Dicer-like1 (DCL1) in a two-step process. The initial cleavage is from the base of the hairpin towards the loop and the second cut is at the loop of the duplex in this canonical miRNA pathway. In an alternative pathway, an initial subset of plant miRNAs are processed from the loop towards the hairpin, followed by cleavage at the base of the hairpin. miRNA/miRNA* duplexes have 5′ monophosphates, two-nucleotide 3′ overhangs, and 3′ ends that are methylated (Me) by HEN1. The duplexes are transported from the nucleus via HASTY, an Exportin5 (Exp5) homolog, for loading into an Argonaute (AGO) complex. (**B**) In animals, canonical miRNAs are processed by the nuclear RNase III enzyme Drosha. The precursor miRNA (pre-miRNA) hairpin is exported to the cytoplasm by Exp5 to generate a single miRNA/miRNA* duplex, which is loaded into a miRNA class AGO protein. In both pathways the miRNA duplex separates into single-stranded miRNA and miRNA*; miRNA∗ is degraded. miRNAs complexes with the AGO RISC complex and guides the complex towards the target transcript. The target mRNA is predominately degraded in plants, and in animals it undergoes translational repression (modified from Axtell et al., 2011).

Primary miRNAs are typically transcribed by RNA polymerase II, followed by capping and polyadenylation (Carthew and Sontheimer, 2009). There are few exceptions to this rule, for example in animals, some miRNAs seem to be transcribed by RNA polymerase III. Most of the miRNAs are generated from

transcription units that produce more than one kind of transcript. miRNA are products of precursor hairpin structures that are processed by RNase III enzymes such as Dicer in plants, Dicer and Drosha family proteins in animals into 21-24 nucleotide sRNAs (Figure. 8). In plants and animals sRNAs associate with Argonaute family proteins to regulate genes at the transcript level (Axtell et al., 2011).

The biogenesis of plant and animal miRNAs is described in Figure 8. In animals, the biogenesis is via the action of two RNase III endonucleases (Drosha) with the aid of dsRNA binding protein (dsRBD). First the pri-miRNA is cleaved into 60-70 nt long pre-miRNAs by Drosha in association with dsRBD (Ghildiyal and Zamore, 2009; Miyoshi et al., 2010). The length of pre-miRNAs are more consistent in animals than in plants, ranging from 55 to 70 nt with the exception of *Drosophila* pre-miRNA which can reach 200 nt in size (Ruby et al., 2007; Axtell et al., 2011). The pre-miRNAs are exported from the nucleus to the cytoplasm with the aid of nuclear export protein Exportin 5 (Yi et al., 2003). Pre-miRNAs are characterized by hairpin structures having two nucleotide overhangs at their 3′ ends. Most of the animal miRNAs are not methylated at the 3′ends. Once in the cytoplasm, Dicer and its interacting partners cleave the pre-miRNA into a duplex containing two strands, the miRNA and miRNA*, corresponding to guide and passenger strand, respectively. The miRNA* is unwound from the duplex and eliminated, while the miRNA associates with an AGO protein to form the miRNA induced silencing complex (miRISC) and guides the complex to the target transcript (Bartel, 2004; Ghildiyal and Zamore, 2009). The regulation of targets by animal miRNAs is through translation repression and in rare cases via mRNA cleavage (Krol et al., 2010).

In plants, the most notable distinction from animal miRNA biogenesis comes from the absence of Drosha for processing the pri-miRNA to the pre-miRNAs. This function is replaced by Dicer-like1 (DCL1). Two sequential cleavage conversions of pri-miRNA to pre-miRNA and finally to miRNA:miRNA* duplex occurs in the nucleus (Millar and Waterhouse, 2005). miRNA biogenesis is completed in the nucleus and the mature miRNA is exported to the cytoplasm with the help of a plant homolog of the exportin-5 protein, HASTY. In plants, the 3′ends of miRNAs are methylated by the HEN1 protein before their export to cytoplasm. HEN1 safeguards plant miRNAs from 3′ uridylation and eventual degradation (Yu et al., 2005; Li et al., 2005). miRNAs in plants regulate their targets by mRNA cleavage and rarely by translation repression.

In plants, most miRNAs originate from the intergenic regions, whereas in animals, they are also derived from introns and coding regions. In humans, 25% of miRNA genes arise from introns (Bartel, 2004). In plants and animals most of the miRNA genes arise from discrete independent transcription units located far off from the targets that they control (Millar and Waterhouse, 2005). Very few plant miRNAs occur as genomic clusters, compared to animal microRNAs. In plants, miRNAs arising from these genomic clusters often give

rise to identical miRNAs as result of tandem duplication events leading to an increase in the dosage effect of a particular miRNA on its targets (Merchan et al., 2009).

There are marked differences between plant and animal miRNA in target recognition. Plant miRNAs often require targets to have perfect or near-perfect complementarity to bind and regulate genes (Llave et al., 2002; Rhoades et al., 2002). In contrast, animal miRNAs need only partial complementarity to pair with targets (Lewis et al., 2003; Rajewsky, 2006). Nonetheless, there are exceptions that break the above-mentioned base pairing rules in both kingdoms. Perfect complementarity, coupled with presence of miRNA-binding motifs in multiple members of gene families, has facilitated accurate prediction of plant miRNA targets. Plant miRNA targets are found in ORFs, 5′ 3′ UTRs and non-protein coding regions (Axtell et al., 2011). In animals and flies, most miRNAs bind with targets to a limited extent at the 5′ ends of the miRNA, "the seed region", leading to translation repression. The seed region is between positions 2 to 7 of the miRNA. This is a major factor that determines target selection and the reason why a single miRNA has multiple targets. The binding sites for animal miRNAs are found usually in 3′UTRs of their target transcripts (Lewis et al., 2003).

miRNAs regulate most biological process in plants and animals (Ghildiyal and Zamore, 2009). The targets are diverse and regulate a variety of processes that includes growth, development, transcription factors, general metabolic pathways, transport pathways, stress responses, and host-microbe interactions (Bartel, 2004; Stefani and Slack, 2008; Katiyar-Agarwal and Jin, 2010).

1.8.8 piRNAs: The hunters of genome invaders

piRNAs are often termed as the vanguard of genome defence, but are so far not found in plants (Siomi et al., 2011). An initial clue about the existence of piRNAs came from an analysis on how *Drosophila stellate* protein coding gene repeats are silenced by sRNAs (Livak, 1990; Aravin et al. 2003). These sRNAs (24-29 nt) were very different from miRNAs and other sRNAs in terms of size, and were mapped to repeated sequences. This finding led to the naming of this new class of sRNAs as repeat-associated small interfering RNAs (rasi-siRNAs) (Aravin et al., 2003). Other studies independently identified a similar class of sRNAs in mammals, which co-immunoprecipitated with Piwi proteins and were designated piRNAs (Liu and Paroo, 2010). There is no potential dsRNA precursor involved in biogenesis of this particular class of sRNAs (Figure 9). piRNAs are 24-30 nt sRNAs which are generated via Dicer-independent pathways and are very distinct from miRNAs or siRNAs. piRNAs in *Drosophila* and mammals carry a 5′ monophosphate and 3′ terminal methyl group and exhibit preference for a 5′ uridine residue (Aravin et al., 2007). There are no signature motifs in piRNA sequences, except for antisense strand piRNAs have strong bias for uridine at postion 1 and sense strand piRNAs have an adenine (A) at position 10. piRNAs map to a relatively small number of genomic regions termed piRNA clusters (Siomi et al., 2011).

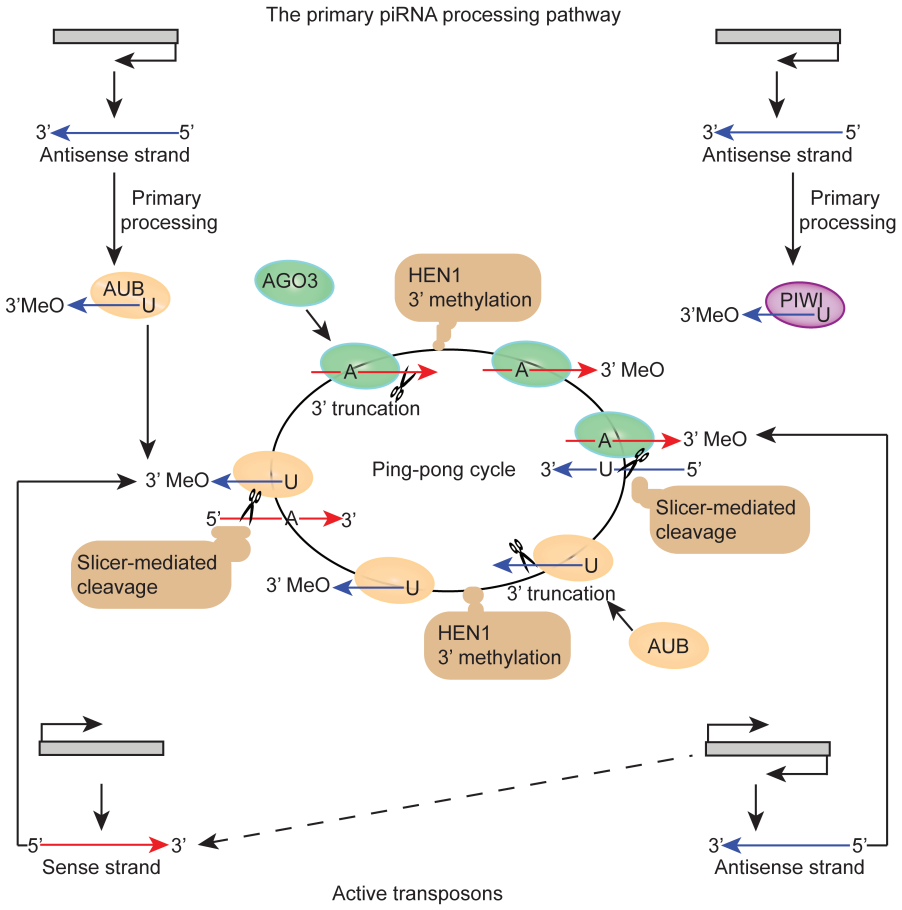


Figure 9. PIWI-interacting RNA (piRNA) pathway. piRNAs are derived from antisense transcripts of transposons and are loaded onto Aubergine (AUB) or PIWI proteins in the primary piRNA pathway. The 3´ends of piRNAs are methylated by HEN1. Antisense piRNA within the AUB complex acts as a progenitor for a ping-pong cycle, cleaving sense precursor transcripts generating sense piRNA that is later loaded on to AGO3. The piRNA bound AGO3 complex cleaves antisense piRNA precursors, generating the 5′ end of antisense piRNAs. These antisense piRNAs are in turn loaded onto AUB. This interdependent cycle continues, generating more piRNAs to regulate transposons (adapted from Siomi et al., 2011).

Large-scale sequencing and genetic studies in flies and mice have suggested two distinct biogenesis pathways: the primary processing pathway and ping-pong amplification loop. These pathways are well conserved across several animal species. The majority of piRNAs are complimentary (antisense) to transposon mRNA and come from the primary processing pathway. piRNAs in the primary pathway associate with PIWI, and Aubergine (AUB); two Argonaute proteins belonging to the PIWI clade. On the other hand, sense-strand piRNAs preferentially associate with Argonaute 3 (Khurana and

Theurkauf, 2010). First, the primary piRNA pathway provides an initial pool of antisense piRNA that target TEs and also serve as trigger for the ping-pong cycle. Next, the ping-pong loop further amplifies the silencing signal by creating more antisense and new sense piRNAs that target more active TEs (Aravin et al., 2003; Siomi et al., 2011). In the primary processing pathway, piRNA precursors are single stranded and formation of primary piRNAs from these precursors in not clearly defined. The size of the piRNAs depends on the specific PIWI protein that they associate with, as the PIWI proteins act as rulers that define the size of mature piRNAs. For example, piRNA that associate with MILI, MIWI2 and MIWI in mice have sizes of 26 nt, 28 nt and 30 nt, whereas in *Drosophila* 25 nt, 24 nt and 23 nt are linked to PIWI, AUB and AGO3 respectively (Siomi et al., 2011).

In the ping-pong amplification loop, the AUB-bound piRNA from the primary pathway directs cleavage of sense-strand mRNAs from active transposons, generating a 5′ end of a new secondary piRNA (Figure 9). Further, these sense strand secondary piRNAs bind to Argonaute 3 (AGO3) and cleave antisense piRNA precursors to produce antisense strand piRNAs that associate with AUB and PIWI. In this amplification loop, the pool of sense and antisense piRNAs that target transposons will be amplified (Aravin et al., 2007; Siomi et al., 2011). Piwi proteins adopt a different silencing strategy for transposon control in comparison to other sRNAs. Studies in diverse animal species using Piwi mutants have shown that Piwi silencing pathways are dedicated to transposon regulation and partly in DNA methylation which may be exerted directly or indirectly (Aravin et al., 2007).

1.8.9 tRNA-derived small RNAs (tRFs)

Another new class of sRNAs that will be briefly discussed here are sRNAs that map to tRNAs. Deep sequencing technologies have always identifed an abundant class of sRNAs derived from non-coding RNAs such as rRNA and tRNA, which were earlier considered as degradation products. Recent studies have shown that tRFs are genuine sRNAs derived via Dicer-dependent and independent pathways (Kawaji et al., 2008; Nunes et al., 2011b) as shown in Figure 10. It has been proposed and proven that tRNA cleavage into tRFs occurs during conditions such as cellular resting stage, stress, and starvation to downregulate or inhibit protein synthesis. This shows that tRFs are part of evolutionarily conserved mechanisms rather than just degradation products (Sobala and Hutvagner, 2011). tRFs have been identified in a number of species ranging from animals to fungi and plants. These sRNAs originate from either the 5′ or 3′ ends of mature tRNAs and the 3′ end of pre-tRNA. They vary in size range of 19 to 40 nt. Most of the tRFs identified in sequencing studies are sense reads. tRFs discovered in humans fall under two classes. Type I tRFs are processed by Dicer dependent pathway in the cytoplasm and down regulate target transcript in trans. Type II tRFs are processed by RNase Z in the nucleus and cleavage takes place close to the 3′ end of tRNA cloverleaf structure (Nunes et al., 2011b).

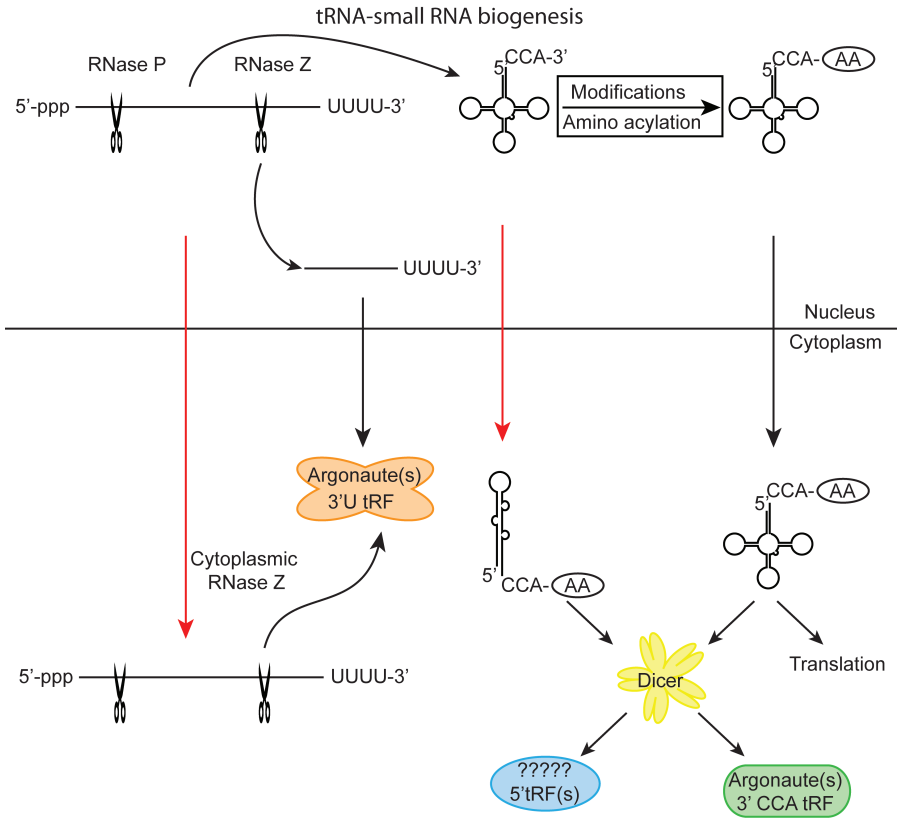


Figure 10. Biogenesis of transfer-RNA-derived fragments (tRFs). RNase Z processes pre-tRNA to 3´tRFs and are exported into the cytoplasm where they are incorporated into Argonaute complexes. Premature pre-tRNAs can also be exported to the cytoplasm and be processed by cytoplasm RNase Z. Alternatively tRNA processed by RNase P and RNase Z followed by addition of terminal CCA may be prematurely exported into the cytoplasm and processed by Dicer. Another possibility that exists is that mature tRNA can enter the cytoplasm and be processed by the Dicer pathway, producing 5´tRNA and 3´ CCA tRFs. 3´ CCA tRFs may be incorporated into Ago complexes. Red arrows indicate possible premature nuclear export (adapted from Sobala and Hutvagner, 2011).

2 Aims of the study

This project is part of a larger Swedish initiative that has the overall objective of decreasing the heavy fungicide burden that potato growers are forced into using, due to the otherwise insurmountable problems with late blight disease. By learning how this pathogen functions and adapts to a changing environment, the aim is to contribute to new management strategies that are based on molecular understanding. This thesis has an emphasis on enhancing the knowledge of the roles that sRNAs play in the plant host – *P. infestans* interaction. The work comprises characterization of the silencing inventory in *P. infestans*, and elucidation of the endogenous sRNA pathways. This knowledge will be utilized further to examine the cross-talk between host plants and this pathogen.

The specific aims were to:

- Study genes encoding the major components of the RNA silencing pathway in *P. infestans* by bioinformatics and experimental approaches.
- Expand the repertoire of reverse genetics tools available for *P. infestans* by developing simpler vector systems for targeted gene silencing.
- Study endogenous sRNAs homologous to effector-encoding genes and transposable elements and their associated biogenesis pathways in *P. infestans*.
- Identify and characterize miRNAs and their targets.
- Categorize and characterize tRNA related sRNAs.
- Search for sRNA genomic hotspots in *P. infestans* and their effects on gene regulation.
- Study the relation between virulence and sRNAs.

3 Results and Discussion

3.1 Paper I

Gene silencing as such was used for silencing of endogenous genes by over expression of transgenes in Oomycetes (Raffaele and Kamoun 2012), but very little is known about the underlying mechanisms in comparison to plants and animal (Whisson et al., 2008). This work was initiated when the *P. infestans* genome was newly sequenced (Tyler et al., 2006) and very little was known about the repertoire of gene silencing mechanisms in this oomycete. Work commenced with a comparative genomic approach to dissect the components of the gene silencing apparatus using the recently sequenced *P. infestans* genome. In total, 51 genes from nine gene families encompassing Dicer-like (PiDcl1), Argonaute (PiAgo), and RNA-dependent RNA polymerase (PiRdr1), histone deacetylases, histone methyltransferases, DEAD helicases, chromodomain proteins, and a class 1 RNaseIII were identified. However, genes that play roles in DNA methylation, such as cytosine methyltransferases and RNA polymerase IV, were not found in the *P. infestans* genome (Vetukuri et al., 2011a).

3.1.1 Components of RNA silencing in *P. infestans*: The loner Dicer

The analysis of the assembled genome of *P. infestans* revealed a single Dicer-like protein (PiDcl1) with two RNaseIII domains and a dsRNA binding domain. Domain organization-wise, PiDcl1 is related to *S. pombe* Dcr1 and *Neurospora crassa* Dcl1, whereas by sequence similarity using BlastP, PiDcl1 was found to be most similar to DCL4 protein from *A. thaliana*. DCL4 in *A. thaliana* is involved in biogenesis of 21 bp siRNA (Qi et al., 2005). Also identified was the Dicer-like Pirnh5 protein that has a predicted DEAD box RNA helicase domain and a poorly conserved dsRNA-binding domain, but no RNaseIII domains. DEAD box proteins are characterized by the conserved motif Asp-Glu-Ala-Asp (Vetukuri et al., 2011a). Interestingly, SOLiD sequencing of sRNAs in this project revealed multiple size classes of sRNAs.

How a single PiDcl1 generates multiple size class of sRNAs needed to be investigated further on. There are reports of multiple Dicer proteins involved in different silencing pathways, generating different size classes of sRNAs in higher eukaryotes (Liu and Paroo, 2010). There are four Dicer proteins in *A. thaliana*, all of which catalyze formation of different sized sRNAs: DCL1 generates miRNA, DCL2 – 22 nt sRNAs, DCL3 – 24nt and DCL4 -21nt sRNAs (Garcia-Ruiz et al., 2010). In contrast, mammalian genomes encode only one Dicer that generates both miRNAs and siRNAs (Liu and Paroo, 2010). It was speculated that this may also be the case in *P. infestans*, although it remains to be experimentally determined.

Of the five Argonaute proteins (PiAgo1-5) mined by comparative genomics, two of them PiAgo1, 2 are identical and have a nuclear localization signal (NLS). Potentially being nuclear-localized, are Argonaute 1\2 involved in transcriptional silencing in *P. infestans*? In plants, Argonautes, apart from their role in mRNA targeting in the cytoplasm, also take part in DNA methylation. On the other hand, in *S. pombe* where DNA methylation is lacking, AGO1 is involved histone methylation events (Ender et al., 2010). In plants and animals, sRNAs are sorted into different Argonaute complexes based on the Dicer involved, the structure of the sRNA duplex, the 5´terminal nucleotides, and thermodynamic properties (Czech and Hannon, 2011). Whether the five Argonaute proteins in *P. infestans* have distinct roles requires further elucidation.

3.1.2 Position of *P. infestans* RNA dependent RNA polymerase amongst the eukaryotes

PiRdr1 is a very unique protein, with a Dicer-like DEAD box RNA helicase and Rdr domains. The only other Rdr identified so far that has similar domain architecture originates from the slime mould, *Dictyostelium discoideum* (Martens et al., 2002). Due to its apparently diverse domain architecture, PiRdr1 protein sequence was selected for further sequence analysis by assessment of relatedness to Rdr proteins from other organisms. Similar to Dicer-like proteins, Rdr sequences from *P. infestans* and *P. sojae* were closely related, while clustering of sequences from other organisms with the oomycete sequences was poorly supported (Figure 11). In contrast to Dicer-like proteins (Figure 2 in paper I), *P. infestans* and *P. sojae* Rdr sequences formed an outgroup separate to fungal, plant, and nematode sequences.

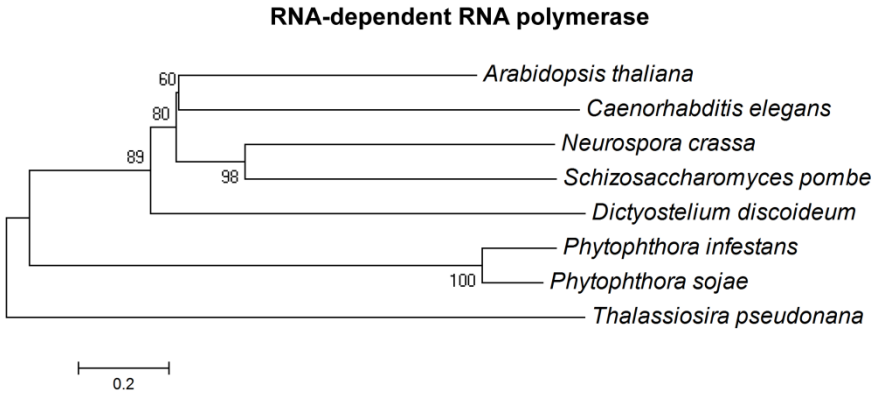


Figure 11. Protein sequence relationships (linearized) for RNA-dependent RNA polymerase proteins. Phylogenetic analyses were conducted in MEGA4 and only predicted functional protein domain sequences of *P. infestans* were used to align with similar sequences in other organisms. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed.

3.1.3 Inquest for a suppressor of silencing: Pirns1 (RNaseIII) does not suppress RNA silencing

By comparative genomics and real-time RT-PCR, the presence of the gene that encodes for Pirns1 protein was determined in *P. infestans*. This protein contains a single RNaseIII domain. It exhibited similarity only to bacterial class 1 RNaseIII proteins at the C-terminus, and represented the simplest class of RNaseIII enzymes. The apparent lack of a clear homolog of enhanced RNAi 1 (ERI1), a ribonuclease that digests siRNAs in *C. elegans* and other organisms and leads to the release of silencing (Kennedy et al., 2004; Iida *et al*., 2006), led to the proposal that *Pirns1* (class I RNaseIII) may be a candidate for this process in *P. infestans*. It has been shown previously that an RNaseIII enzyme of sweet potato chlorotic stunt virus is involved in enhancing RNA-silencing suppression activity of protein p22 (Kreuze et al., 2005) and it is also able to suppress RNA silencing on its own (Cuellar et al., 2009). In this study, *P. infestans* Pirns1 was investigated for its ability to interfere with the silencing of *GFP* in a patch-*Agrobacterium*-infiltration assay in *Nicotiana benthamiana*. In this assay, expression of *GFP* declined by 7 dpi, due to the onset of silencing (Figure 12, seen on A and B, left halves of the leaves). Co-infiltration for expression of *GFP* and potato virus A (PVA) *HC-Pro*, the known silencing inhibitor, resulted in enhanced fluorescence of GFP (Figure 12, seen on B, right halves of the leaf) and higher *GFP* mRNA accumulation (Figure. 12 C), indicative of efficient suppression of RNA silencing. In contrast, co-infiltration of *GFP* with *P. infestans Pirns1* did not result in notable RNA silencing suppression, as revealed by fluorescence (Figure 12, seen on A, right halves of the leaf), Northern and immunoblots (Figure 12 C).

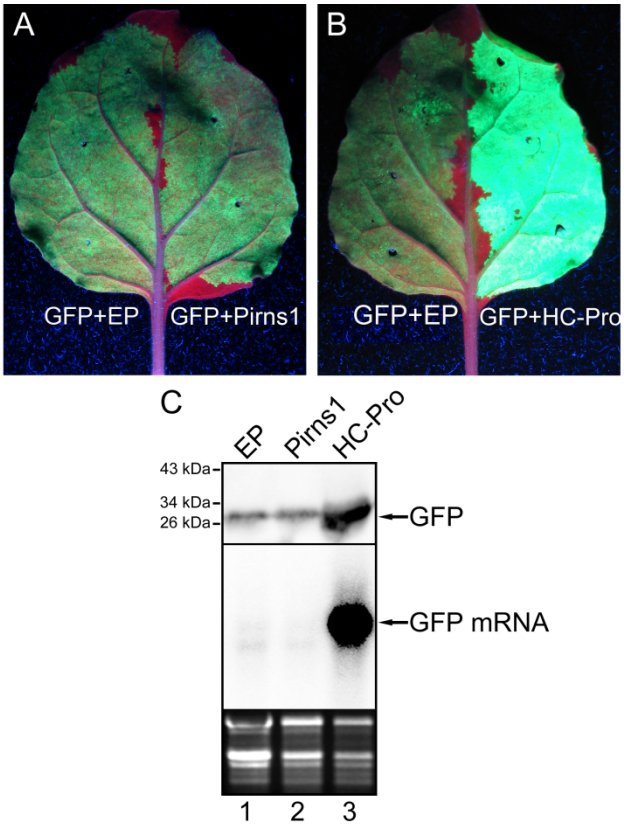


Figure 12. Assay of *P. infestans Pirns1* for possible involvement in suppression of silencing. In panel **(A)** the right half of the *Nicotiana benthamiana* leaf was *Agrobacterium*-infiltrated for expression of *P. infestans Pirns1*. In panel **(B)** the right half of the leaf was *Agrobacterium*-infiltrated for expression of PVA HC-Pro. Left half of the leaves in panel A and B were *Agrobacterium*-infiltrated with pA plasmid without an insert (EP; A and B). All infiltrations were carried out as co-infiltrations with *GFP*. The abaxial sides of the leaves were photographed under UV light at 6 days post infiltration. Bright green fluorescence observed in the half of the leaf infiltrated with HC-Pro is indicative of RNA silencing suppression. Weak fluorescence (A; left part of B image) is due to fluorescence of residual GFP. **(C)** Immunoblot detection of GFP accumulation and Northern blot detection of GFP mRNA in experiments with silencing induced by *GFP* construct. Samples were taken 6 days p.i. The gene constructs co-infiltrated with *GFP* are indicated above the panels. EP = pA plasmid without an insert. *Pirns1*= *P. infestans Pirns1* (RNase III). HC-Pro = helper component proteinase of PVA. Lower panel shows loading of RNA samples.

3.2 Paper II

3.2.1 Junk DNA playing spoiler: Transposon induced gene silencing

Reverse genetics approaches such as targeted gene disruption are not feasible in oomycetes due to the low rate of homologous recombination and the diploid life cycle stages used in laboratory experiments. To overcome these problems,

efforts were made by different labs to exploit homology-dependent gene silencing in oomycetes. Stable silencing in *P. infestans* can be induced by transformation using either sense, antisense or inverted repeats, by either zoospore electroporation or protoplast methods (Ah Fong et al., 2008). The type of gene silencing that operates in stable transformants is reported to be transcriptional gene silencing based on nuclear run-on assays (van West et al., 1999; Judelson and Tani, 2007). Transient silencing can also be attained by treatment of protoplasts with dsRNA homologous to the target gene, which is probably posttranscriptional gene silencing leading to transcriptional silencing (Whisson et al., 2005).

Deep sequencing of different eukaryotic genomes has shown that the majority of genomes are replete with repeats, the majority of which are transposable elements or TEs (Rebollo et al., 2010). Eukaryotic TEs are divided into two major classes based on nature of transposition. The replicative strategy either involves RNA intermediates (class 1 or retrotransposons) or DNA intermediates (class 2 or DNA transposons). Close inspection of different organisms by comparative genomics have shown for instance that 50% of the human genome, 85% of maize, and 14% of the *A. thaliana* genome are composed of TEs (Lander et al., 2001 Schnable et al., 2009; Rebollo et al., 2010). The variations in genome size are attributed to intergenic distances, which are often the result of accumulation and proliferation of transposons, as seen in *P. infestans* (Haas et al., 2009). TEs often contribute to the evolution of the organism in a multitude of ways, most of which are detrimental to the host. However there are many instances where they show dramatic impact on the host, for example, the *Ac/Ds* transposons in maize influence the expression of the neighboring genes, thereby generating a mosaic pattern in maize kernels (McClintock, 1950). TEs, when in close proximity to genes can regulate their expression both at transcriptional and posttranscriptional levels.

The large genome of *P. infestans* is composed of 32.9% *LTR/Gypsy_Ty3*, 3.9% *LTR/Ty1_Copia*, small proportions of many different DNA transposons, and 27% unclassified repeats, all together making up approximately 75% of the entire genome content (Haas et al., 2009). However, *Gypsy* elements such as *Gypsy pi-1* and *Albatross* that are grouped under LTR transposons, alone account for 29 % of the genome. Many eukaryotes employ gene silencing as means to control transposons (Malone and Hannon, 2009), and presumably this may be the case for this pathogen. The *P. infestans* genome has a very unusual bimodal genome architecture consisting of highly conserved gene blocks tightly packed as the core genome, interrupted by transposon-rich regions that are termed the plastic genome (Gijzen, 2009). The effector class genes that are critical to plant infection are predominantly located in the repeat and TE rich region. The effector gene classes such as RxLR are the dynamic force that shapes the pathogen ability to outwit host immune responses. In return, effectors may be influenced by gene silencing mechanisms that control transposons in view of their proximity to these mobile elements (Whisson et al., 2012).

With this background, the aim was to exploit transposon-induced gene silencing as a tool to determine the role of specific genes during infection and to develop a simple tool for gene knockdown in *P. infestans* (II). This work hinted that silencing effects could spread bi-directionally and silence the neighboring genes.

There are over 700 predicted effectors interspersed among the transposons, present at varying distance from the nearest transposons. 563 constitute the RxLR class, and 283 of these genes are located within 2 kb distance from TEs (Whisson et al., 2012). Within this number, 35 RxLRs are located within 300 bp of a transposon-derived sequence, and a total of 106 within a 600 bp range from a TE. The formation of heterochromatin was reported to spread from the point of silenced loci up to 600 bp outward in *P. infestans* (Judelson and Tani, 2007). Deep sequencing data from this project showed that there are thousands of sRNAs that map to transposons (Manuscript III, unpublished data). Based on the experimental evidence on heterochromatin formation, spread and presence of small RNA to transposons, the silencing signal from TEs may spread to the adjacent effector genes. This may be one of the mechanisms by which this particular pathogen benefits from transposons, by suppressing some of the redundant effector genes.

Summarizing the results from gene silencing studies, a model (Figure. 13) can be proposed to show how silencing may spread from transposon to the effector gene based on work with *SINEm-Avr3a* fusions (Paper II).

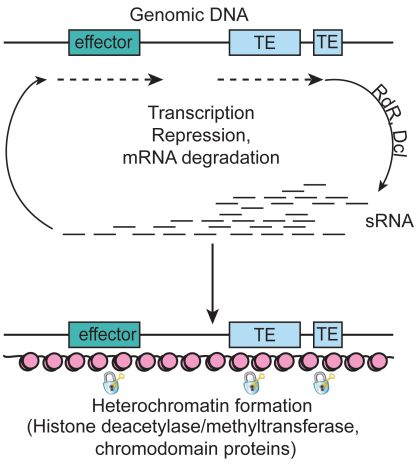


Figure 13. Model for transposon element (TE) induced effector silencing. As part of regulation, TEs in the genome are silenced via transcriptional and post transcriptional silencing through heterochromatin formation and sRNAs targeting TE sequences. The silencing signal from transposon regulation might spread to nearby effector gene either by primary or amplified secondary sRNAs. Amplification of silencing signal is by an Rdr-dependent mechanism. The pink circles in the figure represent histones. The locks on the pink circles represent silent chromatin state (Modified from Whisson et al., 2012).

3.3 Paper III and IV

For *P. infestans*, there are a range of questions that are of significant interest, such as how the expansion of the *P. infestans* genome is regulated, how effector regulation is linked with variation in pathogenicity and specific virulence, whether there are linkages between transposon, gene and effector regulation. To generate some insight into these topics, deep sequencing (Applied Biosystems SOLiD) of sRNAs from two isolates with contrasting infection abilities was performed. From each isolate, RNA was prepared from four life cycle stages; mycelium, sporangium, germinating sporangium and germinating cysts (Manuscript III and IV). Analysis of the *P. infestans* sRNA catalogue revealed the presence of diverse classes of sRNAs originating from different classes of effector encoding genes, transposons, mitochondria, tRNAs, ribosomal RNA, and genomic hotspots. The most abundant size classes of sRNAs were 21, 25/26 and 32 nt. sRNAs of 21nt were the predominant class that mapped to CRN effectors. The existence of the sequenced sRNAs that mapped to different classes of transposons and effector genes were validated by Northerns hybridization. Combined computational and experimental data showed that effector gene-derived sRNAs were present in both isolates, but some exhibited marked differences in abundance. The biogenesis of sRNAs was demonstrated using knockdown of Dicer and Argonaute expression. This has provided evidence of at least two silencing pathways operating in *P. infestans*. One pathways is dependent on Dicer, and the other on Argonautes. Additionally, six miRNA candidates and their targets were identified. These miRNA candidates have features characteristic of both plant and metazoan miRNAs. Overall, the sRNA profiles of the two isolates differed only for relatively few genes and transposons, which are discussed further in manuscript III.

Further profiling of sRNAs classes in *P .infestans* revealed diverse sRNAs that mapped to transfer RNAs, ribosomal RNAs, genomic sRNA 'hotspots', and the mitochondrial genome. Some of these sRNAs have previously been regarded as degradation products, but recent studies have shown that sRNAs originating from tRNAs and rRNA are distinct cleavage products, and have biological function (Sobala and Hutvagner, 2011; Li et al., 2012). Hotspots are defined as small stretches of the genome that generate large numbers of sRNAs, relative to the rest of the genome (Heisel et al., 2008), contributing to modulation of gene function. This type of analysis may be very informative in functional classification of sRNAs. Hotspot analysis in the *P. infestans* genome has revealed over a hundred predicted genes as sRNA hotspots. These corresponded, for example, to CRN effector genes, arrays of gene duplications, potentially antisense overlapping transcripts, and transposon insertions. In

addition, mapping of sRNAs from *P. infestans* isolates to the mitochondrial genome demonstrated that few unique reads aligned exclusively to mtDNA. The presence of these unique reads suggests that there may be an effective RNA silencing mechanism operating for the *P. infestans* mitochondrial genome. Further details are elaborated in manuscript III and IV.

Taken together, these findings demonstrate that the sRNA inventory of *P. infestans* comprises diverse classes other than transposon-derived sRNAs, and provides further evidence that RNA silencing that involves sRNAs may contribute to variability in pathogenicity and specific virulence.

4 Conclusions

These are the main conclusions from the work presented in this thesis:

- All the gene families that are potentially involved in gene silencing pathways were identified by comparative genomics and validated as active genes by real-time RT-PCR. The function of Dicer-like, Argonaute and histone deacetylase proteins were validated for their role in silencing using a reverse genetic approach based on release of transgene induced silencing for a gene necessary for sporulation as a visual readout. In this assay, the sporulation phenotype tracked closely with the lowest levels of transcript from the targeted genes. This indicates that the core components of gene silencing mechanism are functional and *P. infestans* possesses canonical gene silencing pathways similar to other eukaryotes.
- A novel class of 30-40 nt sRNAs were identified that map to actively transcribed short interspersed element (*SINE*) transposons, indicating functional silencing machinery regulating transposons in *P. infestans*.
- Fusion of a *SINE* to a *PiAvr3a* effector gene in *P. infestans* leads to the silencing of both the introduced fusion and endogenous homologous sequences. This can be used a tool for stable silencing to study function of different genes.
- Silencing of RxLR effector PiAvr3a leads to loss of pathogenicity phenotype.
- sRNAs of 21 nt and 25 nt dominate the sRNA landscape in *P. infestans*.
- sRNAs to effector class genes were identified via deep sequencing and validated experimentally. This yields clues towards understanding the link between sRNAs and variability in pathogenicity.
- Knockdown of Dicer and Argonautes highlight the role of these genes in different aspects of RNA silencing pathways.
- The first miRNAs were identified from *P. infestans*, and the presence of mitochondrial, tRNA, and genomic hotspot related sRNAs was also demonstrated.

5 Future perspectives

The study of *P. infestans* assumes great importance in view of its economic damage to crops, estimated to be in the range of billions of dollars worldwide. Gene silencing is an important tool in the study of gene function in *P. infestans*, aiding in the understanding of crosstalk between plant and pathogen. *P. infestans*, apart from its economic importance, can also serve as a new model to study gene silencing mechanisms due to its unique nature. An important question to address, in an evolutionary sense, would be to determine if the silencing in *P. infestans* is most similar to gene silencing in animals or plants.

As continuation of the work presented in this thesis, it will be important to elucidate the function of silencing pathways associated with miRNA, tRNA and the new sRNA classes identified here. Further analysis will be necessary to understand the roles of different Argonautes in Dicer-dependent and independent pathways, and determine the role of RNA dependent RNA polymerase in *P. infestans* RNA silencing. Re-sequencing of sRNAs using Dicer and Argonaute mutants might reveal the groups of Dicer and Argonaute(s)-dependent sRNAs. It is crucial to understand how different sRNA classes are generated in *P. infestans*, as these may be linked to variation in effector gene expression, and hence variation in pathogenicity.

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