Size Matters

RNA Silencing Processes in *Phytophthora infestans*

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Cover: “Potato print” of potato leaves and *P. infestans* sporangia and zoospores
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Size Matters. RNA Silencing Processes in *Phytophthora infestans*

Abstract

Non-coding RNAs do not encode proteins but instead function through their own RNA sequence. These molecules range from several thousands of nucleotides (nt) in length down to around 20 nt. Specifically, small RNAs (sRNAs) have critical functions in eukaryotic cells, despite being only 20-30 nt long. RNA interference (RNAi) is an umbrella term describing gene silencing mechanisms directed by sRNAs bound to Argonaute (Ago) proteins in eukaryotic organisms. While regulatory pathways involving microRNAs, small interfering RNAs and Piwi-interacting RNAs are comparatively well-characterized in plant and animal model organisms, less is known about sRNAs in oomycetes. Yet, characterization of sRNA-directed gene regulation in this group of organisms promises to have important applications, as oomycetes encompass many destructive plant and animal pathogens.

Phytophthora infestans causes late blight of potato and tomato, with worldwide losses in potato production estimated to €5 billion per year. This thesis work identified a diversity of sRNAs in *P. infestans* and described the roles played by Dicer (Dcl) and Ago proteins in gene silencing in this organism. Repetitive elements constitute 75% of the *P. infestans* genome, and accordingly, the majority of identified sRNAs overlapped transposons and repeats. A pathway characterized by 21 nt sRNAs was found to regulate the activity of protein-coding genes and to suppress the activity of an abundant class of transposons comprising *Gypsy* LTR elements. PiDcl1-dependence of 21 nt sRNAs was demonstrated by knockdown of *PiDcl1* and probing for individual sRNAs by Northern hybridization. Downstream of PiDcl1 processing, these 21 nt sRNAs interact with PiAgo1, as evidenced by co-immunoprecipitation and deep sequencing. The other major sRNA class in *P. infestans* was found to be 25 nt long, to be mainly involved in control of transposable elements and to co-purify with PiAgo4.

Endoribonucleolytic cleavage of tRNA into 19-40 nt long fragments was observed in four life cycle stages and during host infection. A role of *PiAgo1* in the tRNA fragment pathway was suggested from knockdown experiments. In addition, host-induced gene silencing (HIGS) was proven functional in the *P. infestans*-potato pathosystem. This strategy was shown to successfully silence four endogenous *P. infestans* genes through expression of RNA silencing constructs in potato during infection. The method could potentially be used as a tool to test candidate pathogenicity genes and to study gene function during different time points of infection. HIGS is a promising technique that could be used to develop potato genotypes with improved late blight defense.

Keywords: Argonaute, miRNA, *Phytophthora infestans*, small RNA, *Solanum tuberosum*, transposon, tRNA

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Dedication

To my grandparents, for all our joyful moments

If you don't understand my silence, how will you understand my words?
Anonymous

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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 RR, **Åsman AKM**, Tellgren-Roth C, Jahan SN, Reimegård J, Fogelqvist J, Savenkov E, Söderbom F, Avrova AO, Whisson SC, Dixelius C. (2012) Evidence for small RNAs homologous to effector-encoding genes and transposable elements in the oomycete *Phytophthora infestans*. *PLoS One* 7: e51399.

II **Åsman AKM**, Vetukuri RR, Jahan SN, Fogelqvist J, Corcoran P, Avrova AO, Whisson SC, Dixelius C. (2014) Fragmentation of tRNA in *Phytophthora infestans* asexual life cycle stages and during host plant infection. *BMC Microbiol.* 14:308.

III **Åsman AKM,** Fogelqvist J, Vetukuri RR, Dixelius C. *Phytophthora infestans* Ago1 binds miRNA and sRNAs from effector genes and transposable elements (manuscript).

IV Jahan SN, **Åsman AKM**, Corcoran P, Fogelqvist J, Vetukuri RR, Dixelius C. (2015) Plant-mediated gene silencing restricts growth of the potato late blight pathogen *Phytophthora infestans*. *J. Exp. Bot*. 66:2785-94.

Papers I, II, and IV are reproduced with the permission of the publishers.

Additional publications
Vetukuri RR, **Åsman AKM**, Jahan SN, Avrova AO, Whisson SC, Dixelius C. (2013) Phenotypic diversification by gene silencing in *Phytophthora* plant pathogens. *Commun. Integr. Biol*. 6:e25890.

The contribution of Anna Åsman to the papers included in this thesis was as follows:

I Performed DNA construct cloning, Northern hybridizations, *Phytophthora* transformations and qRT-PCRs.

II Participated in planning of the project. Except for RNA collection for deep sequencing, AÅ performed all laboratory work, including *Phytophthora* transformations, qRT-PCRs and Northern hybridizations. Analyzed and interpreted the data. Took major responsibility for manuscript writing.

III Planned the major part of the project, performed most laboratory work and wrote the largest part of the manuscript.

IV Participated in planning of the project. Performed parts of the laboratory work; RNA extractions and DNA cloning to generate riboprobes for Northern hybridizations.

Abbreviations

Ago	Argonaute
Cas	CRISPR-associated
CNL	Coiled-coil, nucleotide binding site, leucine-rich repeat
CRISPR	Clustered regularly interspaced short palindromic repeats
CRN	Crinkler
Dcl	Dicer-like
HIGS	Host-induced gene silencing
hp	Hairpin
miRNA	MicroRNA
ncRNA	Non-coding RNA
nt	Nucleotides
piRNA	Piwi-interacting RNA
PTGS	Post-transcriptional gene silencing
RdDM	RNA-directed DNA methylation
RdRP	RNA-dependent RNA polymerase
RNAi	RNA interference
sgRNA	Single guide RNA
RxLR	Arginine any-amino-acid leucine arginine
siRNA	Small interfering RNA
sRNA	Small RNA
TE	Transposable element
TGS	Transcriptional gene silencing
TNL	Toll interleukin-1 receptor, nucleotide binding site, leucine-rich repeat
tRNA	Transfer RNA

1 Introduction

The study of small RNA (sRNA) molecules is a rather new research area that started off in the 1990's, with the discovery of small interfering RNA (siRNA) and microRNA (miRNA) in plants and animals (Fire *et al.*, 1998; Waterhouse *et al.*, 1998; Hamilton and Baulcombe, 1999). Bacterial sRNA-mediated regulation was described already in 1981, when two antisense sRNAs were shown to control plasmid copy number (Stougaard *et al.*, 1981; Tomizawa *et al.*, 1981). Today, sRNAs are considered as universally expressed key components of nucleotide-specific gene regulatory systems. The discovery of siRNAs has laid the basis for the development of new molecular tools to suppress gene expression in a wide range of organisms. Meanwhile, the identification of miRNAs has increased our understanding of endogenous gene regulation.

sRNA research has seen great progress in the last few years, much due to improvements in DNA sequencing methods. New high-throughput sequencing technologies have considerably increased the number of sRNA samples that can be analyzed in parallel (Raabe *et al.*, 2014). With decreased prices, genome-wide sRNA expression studies are feasible. Furthermore, the speed of sRNA data generation has been significantly accelerated. Thanks to deep sequencing, it is now possible to discover new, low-abundance sRNA species, which in earlier studies were overshadowed by sequence reads from highly expressed RNAs.

There are many common denominators between plant defense and sRNA biology. RNA silencing was early on recognized as an antiviral defense mechanism in plants (Baulcombe, 1996). Later findings identified the miRNA system as an important component of the plant immune response (Navarro *et al.*, 2006). Conversely, viruses, bacteria and oomycetes use RNA silencing suppressors to subvert the host defense and cause infection (Pumplin and Voinnet, 2013). Clearly, to advance our knowledge of plant disease and resistance, it is important to understand the function of sRNAs.

sRNA biology and plant immunity are two large and active research fields. It has not been possible for me to cover all aspects related to these two topics in this thesis summary. I have chosen to focus on literature describing the basic biology of oomycetes, plant resistance and sRNAs, in combination with publications describing recent findings. The aim was to put these two subjects into an evolutionary context and to briefly describe sRNAs in organisms other than plants and oomycetes. Additional information can be found in cited review articles.

1.1 Small non-coding RNAs

1.1.1 Regulatory RNA

Non-coding RNAs (ncRNAs) lack protein-coding capacity, but many of them have regulatory functions within the cell. Regulatory RNAs vary in length, from several kilobase pairs (kb) down to a few nucleotides (nt), and are critical to all known life forms. The ones less than 200 nt in length are referred to as sRNAs (Clark *et al.*, 2013). A large diversity of sRNAs has been discovered in recent years, and they have been identified in organisms from all domains of life. Common to all sRNAs is that they carry out their regulatory function through base pairing with target nucleic acids. They can be divided into many different classes, based on criteria such as their biogenesis mechanism, size, 5´ nt identity, Argonaute (Ago) binding partner and function (Claycomb, 2014).

Some sRNAs are common to all organisms, such as transfer RNA (tRNA) and 5S ribosomal RNA (rRNA; Mallick and Ghosh, 2012). Other sRNAs are domain-specific, for example eukaryotic spliceosomal RNAs (snRNAs) and siRNAs, and bacterial sRNAs. Small nucleolar RNAs (snoRNAs) are shared by eukaryotes and archaea, and CRISPR RNAs (crRNA; Rath *et al.*, 2015) are common to archaea and bacteria.

1.1.2 Small interfering RNA

Eukaryotic sRNAs produced from long double-stranded RNA (dsRNA) or from RNA with large stretches of self-complementarity are termed siRNAs (Claycomb, 2014). This large and diverse sRNA class is widespread among eukaryotic organisms. siRNAs are typically 20-26 nt long and derive from both endogenous and exogenous sources. Endogenous (endo-) siRNAs are generated from repetitive sequences such as pseudogenes and transposable elements (TEs), from self-complementary RNAs (inverted repeats), or from products of convergent transcription. Additional sources of endo-siRNAs exist in organisms that encode RNA-dependent RNA polymerase (RdRP). Endo-siRNAs play important roles in transposon silencing by preventing TEs from causing insertional mutagenesis (Malone and Hannon, 2009).

Exogenous siRNAs are products of viral or transgene RNA, and act to silence their source RNAs by guiding Ago-mediated cleavage (Pyott and Molnar, 2015). The process of exogenously induced siRNA-directed silencing was the original meaning of the term RNA interference (RNAi). This expression is now used in a much broader context, to include all sRNA-mediated silencing processes in which Ago and Dicer (Dcl) proteins are involved (Claycomb, 2014). siRNAs can be further divided into many subclasses, which are thoroughly described in recent reviews (Axtell, 2013; Martinez de Alba *et al.*, 2013; Bologna and Voinnet, 2014). The siRNA pathway in plants is illustrated in Figure 1.

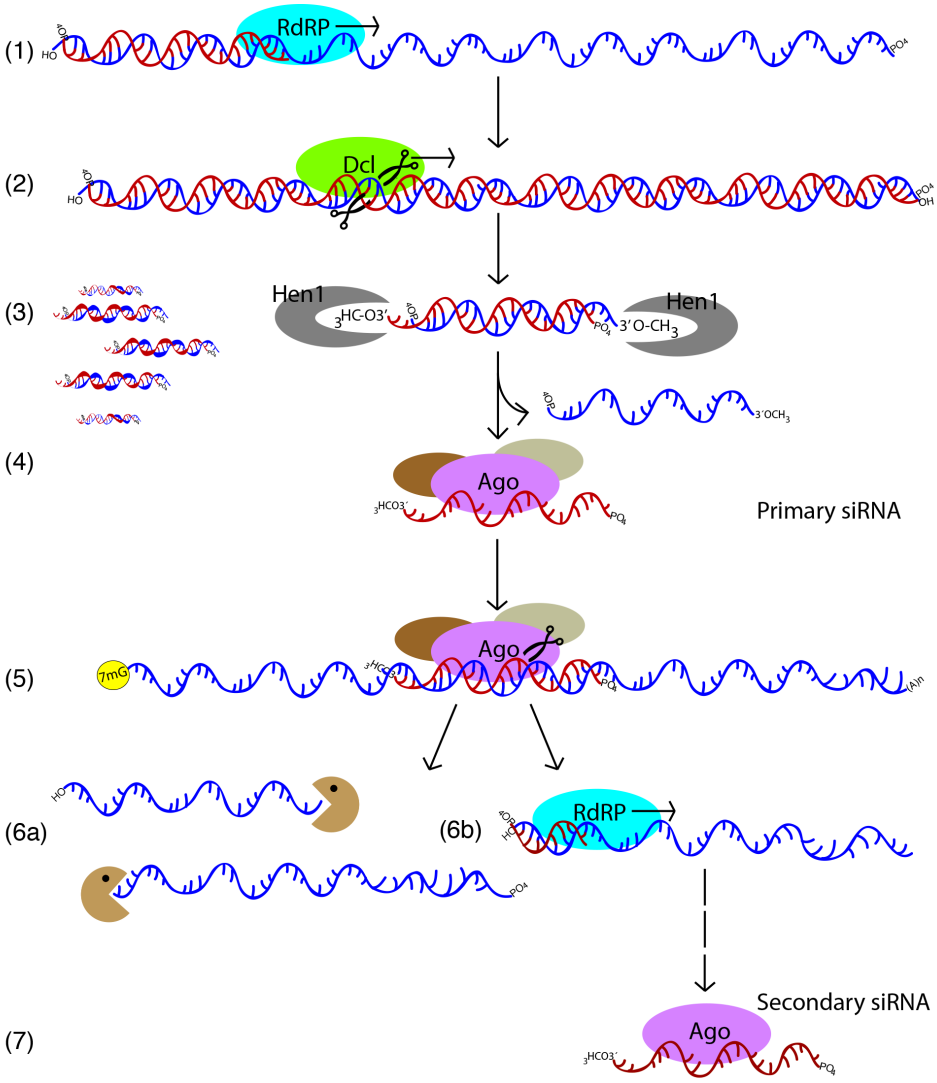

Figure 1. Schematic plant siRNA pathway. (1) RdRP uses single-stranded RNA as template to produce perfectly complementary dsRNA. Examples of alternative sources of dsRNA are viral replication intermediates, products from convergent transcription and experimentally introduced hairpin constructs. (2) Dcl cleaves both strands of the dsRNA and generates several siRNAs as it translocates along a long dsRNA. (3) Hen1 2´-O-methylates both 3´ ends of the siRNA duplex. (4) The guide strand is loaded into an Ago complex and the passenger strand is discarded. (5) Plant siRNAs are highly complementary to their targets and direct Ago-mediated target cleavage. (6a) The cleavage products are degraded by 5´-3´ or 3´-5´ exonucleases. (6b) Alternatively, the cleavage products may serve as templates for RdRP that generates new Dcl substrate dsRNA. (7) Secondary siRNAs amplify the silencing process. Modified from Ghildiyal and Zamore (2009), Christie *et al.* (2011) and Pyott and Molnar (2015).

1.1.3 MicroRNA

Unlike siRNAs, miRNAs are encoded by their own dedicated genes. These genes are transcribed by RNA polymerase II into primary miRNA (pri-miRNA) transcripts, from which mature miRNAs are processed (Czech and Hannon, 2011). So far, miRNAs have been identified in four eukaryotic kingdoms: Mycetozoa (in *Dictyostelium discoideum*), Stramenopila (in oomycetes, a brown alga and a diatom), Viridiplantae (in numerous plants) and Metazoa (in numerous animals; www.mirbase.org). In addition, some viruses encode miRNAs. The last eukaryotic common ancestor (LECA) probably possessed a basic RNAi machinery that acted in defense against viruses and transposons. miRNAs, on the other hand, have evolved independently in different eukaryotic lineages. This assumption is based on (i) the differences in miRNA biogenesis, structure and mechanism of action between animals and plants, (ii) the lack of plant-animal conserved miRNAs and (iii) the absence of miRNAs in many eukaryotic lineages (Cerutti and Casas-Mollano, 2006; Shabalina and Koonin, 2008).

Typically, miRNA maturation in plants and animals involves the precise processing of a long hairpin-containing pri-miRNA transcript into an intermediate structure, called a precursor miRNA (pre-miRNA; Czech and Hannon, 2011). The pre-miRNA is in turn cleaved into a 20-24 nt long miRNA/miRNA* duplex. In this structure, the miRNA strand is referred to as the predominantly expressed sRNA and the miRNA* as the complementary sRNA generated from the opposite arm of the hairpin precursor (www.mirbase.org). In animals, the generation of a miRNA/miRNA* is carried out in two steps, mediated by two ribonuclease III (RNase III) enzymes. The nuclear-localized Drosha makes the first cut. The second step takes place in the cytoplasm, where Dcl removes the terminal loop from the pre-miRNA (Figure 2). Some animal miRNAs ("mirtrons") are processed from spliced-out introns rather than from pri-miRNAs and therefore do not require Drosha cleavage. Animal pre-miRNAs are typically 60-70 nt (Czech and Hannon, 2011), while plant pre-miRNA are much more variable in size, ranging between around 50-900 nt (Bologna and Voinnet, 2014). Plant miRNA maturation requires two to four cleavages, which are all carried out by a nuclear Dcl enzyme. After export from the nucleus, 2´-*O*-methylation at the 3´ end protects plant miRNA/miRNA* duplexes from degradation.

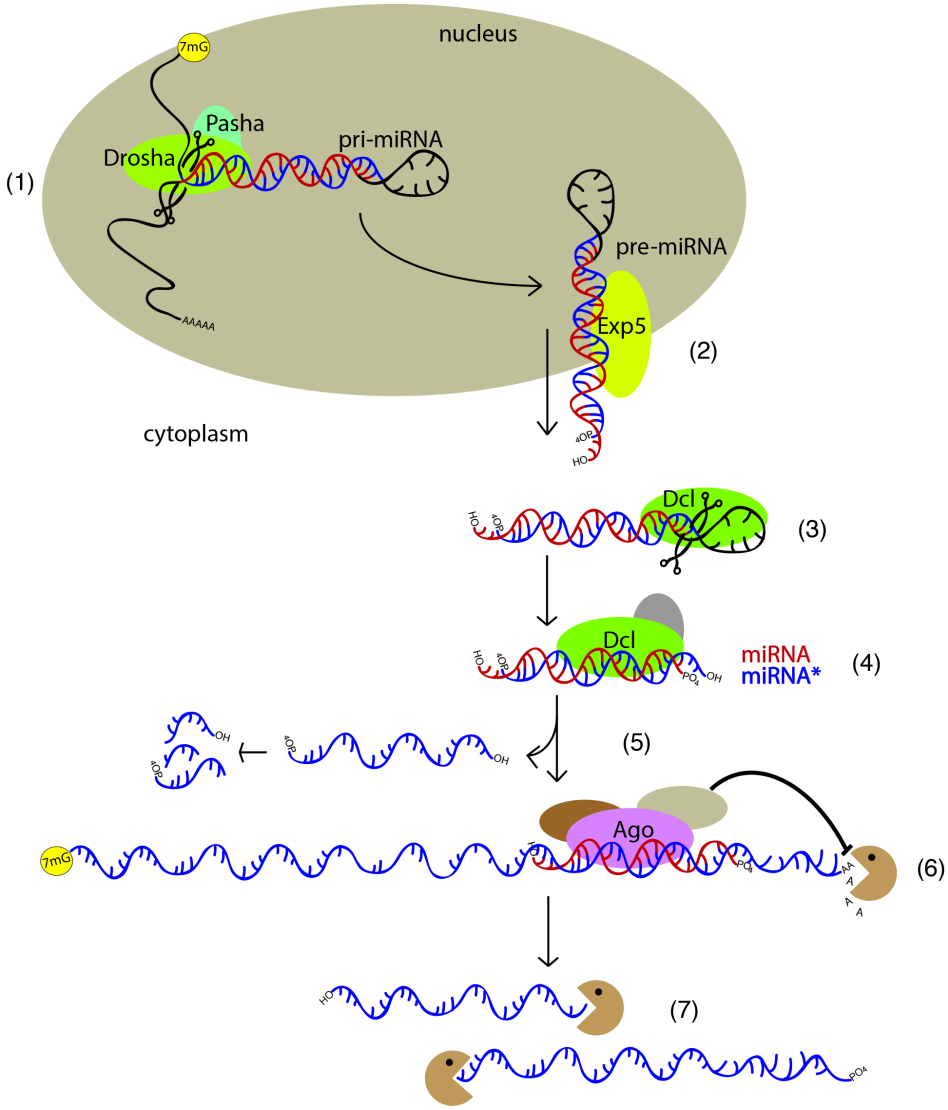

Figure 2. Schematic animal miRNA pathway. (1) pri-miRNA transcripts are capped and polyadenylated products of RNA polymerase II. In the canonical miRNA pathway, a nuclear complex containing the RNase III enzyme Drosha removes the single-stranded tails from the pri-miRNA and liberates a pre-miRNA hairpin. Alternative biogenesis mechanisms include the mirtron pathway, in which pre-miRNAs are generated from intron lariat structures independently of Drosha. (2) Exportin-5 (Exp5) mediates export of the pri-miRNA through the nuclear pore, after which (3) Dcl-mediated removal of the hairpin loop creates a (4) miRNA/miRNA* duplex. (5) The miRNA is incorporated into an Ago complex and the miRNA* is degraded. (6) Animal miRNAs typically have partial complementarity with their targets and bind in mRNA 3´ UTR regions. (7) They induce silencing by deadenylation and decapping followed by mRNA decay. Modified from Huntzinger and Izaurralde (2011) and Meister (2013).

1.1.4 Piwi-interacting RNA

The piRNA class of sRNAs is named after its interacting Piwi-subtype Ago proteins. piRNAs are typically longer (23-30 nt) than siRNAs and miRNAs, are Dcl-independent and derive from single-stranded RNA precursors (Sato and Siomi, 2013; Chak and Okamura, 2014). They are expressed specifically in animal gonads, where their role is to protect the germline from potentially harmful activity of transposons. Some piRNAs also target protein-coding genes (Claycomb, 2014). The biogenesis and action of piRNAs are described in Figure 3.

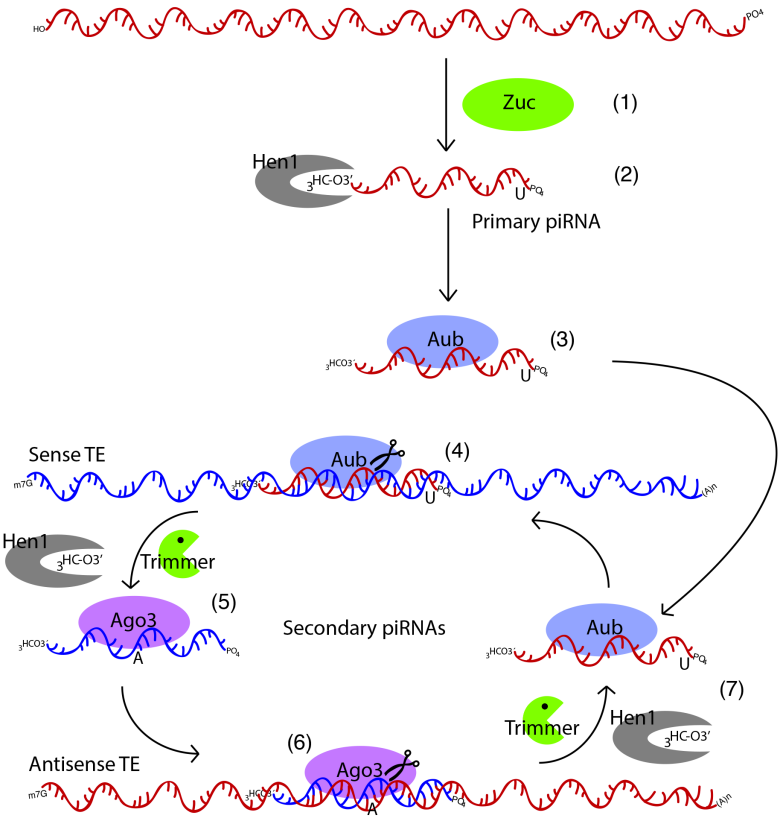

Figure 3. A model of the piRNA pathway in *Drosophila melanogaster*. (1) In the primary processing pathway, piRNA precursors are transcribed from TE-encoding piRNA clusters, processed into piRNAs by Zucchini (Zuc) and (2) methylated by Hen1. (3) The Piwi protein Aubergine (Aub) has a preference for piRNAs carrying 5′ U (1U piRNAs). (4) Guided by antisense piRNAs, Aub cleaves sense TE transcripts. (5) Secondary piRNAs are generated from the cleavage product through 3′ end trimming (Trimmer) and methylation (Hen1). Since Ago proteins cleave their targets between position 10 and 11 of the guide, piRNAs formed from 1U piRNA-directed cleavage will have A at position 10. (6) Such 10A piRNAs guide slicing of antisense TEs by the Piwi protein Ago3. This feeds the ping-pong amplification loop by creating precursors for (7) antisense piRNAs. Modified from Sato and Siomi (2013) and Meister (2013).

1.1.5 Prokaryotic sRNAs

Short RNA molecules also play important roles in prokaryotes. Being typically over 50 nt, bacterial sRNAs are longer than eukaryotic miRNAs, siRNAs and piRNAs (Michaux *et al.*, 2014). Broadly, bacterial sRNAs can be classified into two groups; those that act by antisense inhibition (base-pairing to other RNAs) and those that bind to regulatory proteins (Mallick and Ghosh, 2012). Bacterial sRNAs commonly act at the post-transcriptional level, but transcriptional regulation is obtained by antisense interactions with mRNAs encoding transcription factors or by binding to transcriptional regulatory proteins (Holmqvist *et al.*, 2010). The genome of a given gram-negative bacterium is estimated to encode 200-300 sRNAs, which regulate diverse processes such as plasmid replication, transposon activity, phage life cycles and bacterial metabolism (Mallick and Ghosh, 2012; Michaux *et al.*, 2014). Similar to their eukaryotic counterparts, bacterial sRNAs have primarily a repressive effect on their target mRNAs. Some bacterial sRNAs however stabilize the bound mRNAs or activate their translation.

Recent findings suggest that bacteria also produce shorter sRNAs (15-19 nt) which, similarly to eukaryotic sRNAs, are bound by Ago proteins (Olovnikov *et al.*, 2013). This is described further in chapter 1.2.3. Another class of prokaryote-specific sRNAs is the clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA (crRNA). Together with CRISPR associated (Cas) proteins, crRNAs form the core of a prokaryotic adaptive immune system that act to silence foreign nucleic acids (Rath *et al.*, 2015).

1.1.6 CRISPR/Cas in gene engineering

In recent years, the CRISPR/Cas system has been employed as a tool for gene disruption and modification in prokaryotic and eukaryotic cells (Sander and Joung, 2014). The most commonly used CRISPR-based genetic engineering technique uses *Streptococcus pyogenes* Cas9 and a crRNA derivative called sgRNA (single guide RNA; Ran *et al.*, 2013). Like Ago proteins, Cas9 is a sRNA-guided nuclease, but Cas9 cleaves DNA instead of RNA. The cleavage specificity of the sgRNA/Cas9 complex is determined by 20 nt at the 5′ end of sgRNA (Sander and Joung, 2014). Either one of two pathways of the cellular DNA repair system is exploited to induce genomic mutations; non-homologous end joining (NHEJ) or homology-directed repair (HDR). While NHEJ is error-prone and induces random indels at the target locus, HDR is highly specific, and can be employed to create precise amino acid changes or replace large gene fragments.

The targeting specificity of Cas9 can easily be changed, by the design of a new pair of DNA oligos and their incorporation into the sgRNA backbone (Ran *et al.*, 2013). Thus, the CRISPR/Cas9 technique is highly flexible and relatively cheap. In addition, plasmids, protocols and many design tools are freely available online (Bortesi and Fischer, 2015). The method is applicable on a wide range of biological systems and has been shown functional in e.g. bacteria, yeast, human cell lines, roundworm, wheat (Sander and Joung, 2014) and Cynomolgus monkey (a primate model species; Niu *et al.*, 2014). The technique can easily be multiplexed, by co-delivery of several sgRNAs into the target cell, and it is possible to generate modified organisms without foreign DNA (Bortesi and Fischer, 2015). This was demonstrated in rice, where Cas9 and sgRNAs segregated away from the modified genomic locus in the progeny of self-fertilized transgenic plants (Zhou *et al.*, 2014). The drawbacks of CRISPR/Cas9 are the limited knowledge on potential off-target effects and the requirement for a Cas9 recognition sequence (NGG) downstream of the target sequence (Ran *et al.*, 2013).

1.1.7 Regulatory mechanisms of sRNAs

RNA gene silencing operates either at the post-transcriptional or transcriptional level (referred to as PTGS and TGS, respectively). Common to both mechanisms is the central role of sRNAs and Ago proteins, acting as guides and executors of the silencing process, respectively. Depending on the degree of base pairing between the sRNA and the target and on the nature of the Ago involved in the process, PTGS works through one of the following pathways: (1) target cleavage, (2) exoribonucleolytic degradation in the 5′-3′ and 3′-5′directions or (3) translational inhibition (Czech and Hannon, 2011; Huntzinger and Izaurralde, 2011). Cleavage of target RNA (slicing) dominates for catalytically active Ago proteins bound by sRNAs with extensive complementarity to the target. This mechanism is typical of plant Ago proteins and the siRNA pathway in animals (Czech and Hannon, 2011). RNAs targeted by a mismatched sRNA bound by a non-catalytic Ago are typically repressed through a pathway involving deadenylation, decapping and 5′-3′decay. Translational inhibition has been regarded as the major contributor to miRNA-directed silencing in animals, but recent studies show that mRNA decay dominates on a genome-wide level in mammalian cells (Huntzinger and Izaurralde, 2011; Eichhorn *et al.*, 2014). For sRNAs with partially complementary targets, typical of animal miRNAs, extensive base pairing in the “seed” region (nt 2-8 of the sRNA) is critical for target recognition (Axtell *et al.*, 2011). Animal miRNA binding sites are located in the mRNA 3′

untranslated regions (UTRs), while target sites of plant miRNAs can be located anywhere in the mRNA.

TGS is induced by a sRNA-Ago guide complex through recruitment of DNA methyltransferases or repressive chromatin modifiers such as histone deacetylases and histone methyltransferases (Sabin *et al.*, 2013). As in PTGS, the identity of the target locus is specified by sequence homology to a sRNA. Transcriptional silencing mainly acts to silence TEs, but it also functions in epigenetic inheritance, intercellular communication and stress responses (Castel and Martienssen, 2013, Matzke and Mosher, 2014). In plants, DNA methylation is induced through a process called RNA-directed DNA methylation (RdDM), which is described in 1.6.2.

1.2 Core proteins required for RNAi

1.2.1 Dicer and RNA-dependent RNA polymerase

A large number of proteins are involved in sRNA biogenesis and in execution of RNA silencing. The most highly conserved and most central RNAi proteins will be covered in this chapter.

Biogenesis of the majority of siRNAs and miRNAs require Dcl proteins. Dcls are RNase III enzymes, and as such, specifically cleave dsRNA (Court *et al.*, 2013). The dsRNA substrates can be formed from intermolecular sense-antisense hybrids (e.g. viral replication intermediates) or from partially self-complementary single-stranded RNAs (e.g. pre-miRNAs; Czech and Hannon, 2011). Human and *Arabidopsis thaliana* Dcls are multidomain proteins composed DExD helicase, DUF283, PAZ (Piwi-Argonaut-Zwille), 2x RNase III and dsRBD domains (Bologna and Voinnet, 2014; Kurzynska-Kokorniak *et al.*, 2015). Many eukaryotic Dcl enzymes however lack one or more of these domains. For example, *Giardia lamblia* Dcl is composed of a PAZ and dual RNase III domains, and *P. infestans* Dcl1 does not have a PAZ or a dsRBD domain (Macrae *et al.*, 2006; Vetukuri *et al.*, 2011a). Dcl enzymes contain a single catalytic center, wherein the two RNase III domains cut one RNA strand each (Kurzynska-Kokorniak *et al.*, 2015). The cleavage reaction generates a sRNA duplex carrying a 5′ phosphate and a 2-nt 3′ overhang at both ends, the hallmark of RNase III-dependent cleavage (Czech and Hannon, 2011). The length of the sRNA product is determined by the distance between the PAZ domain, which binds the end of the dsRNA substrate, and the catalytic center (Macrae *et al.*, 2006). Due to structural difference between Dcl proteins, different organisms have distinctive sRNA size profiles. The N-terminal helicase domain is involved in mediating processivity, i.e. allowing Dcl to produce successive sRNAs as it moves along a substrate (Lau *et al.*, 2012).

In organisms possessing an RdRP, RNA silencing can be amplified by the production of secondary sRNAs. The amplification process in *A. thaliana* typically starts from Ago cleavage products, which are used by RdRPs to produce dsRNA (Bologna and Voinnet, 2014). Dcl proteins then cleave the dsRNAs into secondary siRNAs. *Caenorhabditis elegans* RdRPs generate secondary siRNA directly, without the need of Dcl or a primer. In contrast to plant secondary siRNAs, the worm equivalents are produced as single stranded entities and have 5′ di- or triphosphates (Sijen *et al.*, 2007). *C. elegans* secondary siRNAs can trigger the generation of a third wave of siRNAs. These tertiary siRNAs are involved in transgenerational silencing and paramutation (Sapetschnig *et al.*, 2015). The latter concept involves the induction of a heritable change in expression level at one allele caused by the homologous allele without any mutation in the DNA sequence.

Eukaryotic RdRPs have a monophyletic origin (Cerutti and Casas-Mollano, 2006). Notably, the genomes of vertebrates and insects do not encode RdRP homologs, and RdRP activity has not been detected in mammalian cells (Stein *et al.*, 2003; Zong *et al.*, 2009). Yet, these organisms have intricate sRNA-based silencing pathways, comprising siRNAs, miRNAs and piRNAs. In addition, *Drosophila* has the capacity to mount a systemic antiviral RNAi response (Saleh *et al.*, 2009). This implies (i) that RdRP has been lost in these animal lineages, and (ii) that sRNA amplification by RdRP is not required for efficient RNA silencing. Possibly, amplification of siRNAs through transitive RNAi (i.e. the generation of secondary siRNAs 5′ and 3′ of the initial siRNA target site) is required only in organisms relying on siRNA spreading between cells and tissues. The finding of systemic silencing in flies (Saleh *et al.*, 2009) however challenges this idea.

1.2.2 Argonaute

To induce RNA silencing, sRNAs need to associate with a protein of the Ago superfamily. The so-called minimal RNA-induced silencing complex (RISC) is composed of an Ago complexed with a sRNA. RISC commonly includes additional proteins, but a purified Ago-sRNA complex is sufficient for silencing (Rand *et al.*, 2004; Rivas *et al.*, 2005).

X-ray crystallography has enabled detailed characterization of the interaction between Ago proteins and sRNAs. Prokaryotic Agos (pAgos) were the first to be crystallized: archaeal Ago from *Pyrococcus furiosus* and bacterial Ago form *Aquifex aeolicus* (Song *et al.*, 2004; Yuan *et al.*, 2005). More recently, structures of yeast Ago and human Ago2 were also obtained (Nakanishi *et al.*, 2012; Schirle and MacRae, 2012). The domain architecture is highly conserved between these four organisms. The function of the N-terminal

domain is not fully understood, but it seems to play roles in sRNA duplex unwinding and target slicing (Kwak and Tomari, 2012; Faehnle *et al.*, 2013). Positioned next to the N-terminal domain is the PAZ domain, which binds the sRNA 3′ end nucleotide. The Mid domain harbors the 5′ nucleotide-binding pocket and the nucleotide specificity loop, the latter sensing the identity of the 5′ base (Frank *et al.*, 2010). The most C-terminal part is made up by the PIWI domain, which mediates endoribonucleolytic activity ("slicing") in catalytic Agos. The PIWI domain adopts an RNase H fold, which contains four conserved catalytic residues (Asp, Glu, Asp, Asp/His) in Ago proteins capable of target cleavage (Faehnle *et al.*, 2013). This motif is however not sufficient for slicing, since all Agos having an intact catalytic tetrad are not catalytically active

1.2.3 Origin of eukaryotic RNAi

The three main protein components (Dcl, RdRP, Ago) involved in RNA silencing have deep eukaryotic roots. This suggests that the common ancestor of all eukaryotes, LECA, possessed a basic RNAi machinery (Cerutti and Casas-Mollano, 2006). This regulatory system likely acted to defend the early eukaryotic genome against invasive nucleic acids, such as viruses and transposons. Some of the present eukaryotic lineages however lack one or more proteins of the RNAi machinery and are not capable of RNAi (Shabalina and Koonin, 2008). This implies independent losses of this trait during the course of evolution. For example, *Schizosaccharomyces pombe* is a model organism for sRNA-guided heterochromatin formation, but the distantly related yeast *Saccharomyces cerevisiae* does not use RNA silencing (Camblong *et al.*, 2007; Nicolas *et al.*, 2013). Among the Apicomplexa, *Plasmodium falciparum* does not have RNAi, but *Toxoplasma gondii* possesses the three core RNAi protein factors and several classes of sRNAs (Braun *et al.*, 2010; Nicolas *et al.*, 2013). Analysis of non-eukaryotic homologs of Dcl, RdRP and Ago suggests that the three proteins have diverse phylogenetic origins (Shabalina and Koonin, 2008). RdRP has presumably been acquired from viral sequences. The RNase III and helicase domains of Dcl seem to be of bacterial and archaeal origin, respectively. Ago homologs with N-PAZ-Mid-PIWI domain organization are found in all three domains of the tree of life. Eukaryotic Ago proteins are however more similar to archaeal than bacterial Ago homologs (Shabalina and Koonin, 2008; Swarts *et al.*, 2014b). The time of divergence between the two Ago subtypes, Piwi and Ago, is not yet resolved, but the widespread distribution of subfamily members amongst eukaryotic supergroups suggests that already LECA possessed two Ago genes.

Approximately 20% of sequenced bacterial and archaeal genomes contain Ago-encoding genes (Hur *et al.*, 2014), and the first x-ray crystallography studies of Agos were conducted on prokaryotic proteins. Little is known however about the nucleic acid binding partners and functional roles of pAgo proteins. Characterization of pAgos in the archaeon *Pyrococcus furiosus* (PfAgo) and the bacteria *Rhodobacter sphaeroides* (RsAgo) and *Thermus thermophilus* (TtAgo) found that all three proteins are capable of binding short nucleic acids and of targeting homologous sequences for silencing (Hur *et al.*, 2014; Swarts *et al.*, 2015). While RsAgo associated with both 15-19 nt sRNA and 22-24 nt DNA, TtAgo bound only short DNA (13-25 nt). Unlike the eukaryotic Agos engaged in RNAi, pAgo complexes from both bacteria target complementary DNA (Olovnikov *et al.*, 2013; Swarts *et al.*, 2014a). RsAgo-bound sRNAs were enriched for sense transcripts mapping to plasmids, phages and transposons, suggesting a role for the pAgo/sRNA complex in repression of extra-chromosomal DNA elements (Olovnikov *et al.*, 2013). In line with this idea, the gene expression levels from an exogenous plasmid were elevated in an RsAgo mutant strain. Similar to many eukaryotic Ago proteins, RsAgo showed a bias towards sRNA with a 5′ U residue. Eukaryotic Agos are more closely related to archaeal PfAgo than to RsAgo and TtAgo. Nevertheless, PfAgo uses DNA as both guides and targets (Swarts *et al.*, 2015).

1.2.4 Argonaute-dependent sRNAs

Given its central role in sRNA production, Dcl was first believed to be absolutely required for RNAi. New sRNA biogenesis pathways that by-pass the need for Dcl have however recently been discovered (reviewed by Chak and Okamura, 2014). In most cases, these Dcl-independent sRNAs are generated from single-stranded RNA (ssRNA) by Ago-mediated cleavage.

One example of an Ago-dependent sRNA pathway is the piRNA biogenesis mechanism in the animal germline (Sato and Siomi, 2013). Primary piRNAs are generated from bidirectional piRNA clusters, which encode TE sequences on both genomic strands, and fed into the so-called ping-pong loop (Figure 3). Biogenesis of secondary piRNAs through this amplification process requires two germline-expressed Piwi-class proteins, Aub and Ago3. Aub binds 5′ U piRNAs that are antisense to TEs and cleaves sense TE transcripts. New piRNAs, sense to TEs and carrying A at position 10, are formed through 3′ trimming of the cleavage products by an unknown 3′-5′ exonuclease called Trimmer (Kawaoka *et al.*, 2011). The sense piRNAs then guide Ago3 into cleavage of antisense TE transcripts (Sato and Siomi, 2013). This creates an amplification loop, since antisense piRNAs generated from the Ago3-cleaved transcripts allow Aub to again cleave sense TEs.

3′-5′ exonuclease trimming of Ago-bound sRNAs might be a recurrent maturation mechanism of Dcl-independent sRNAs. In fission yeast, formation of centromeric heterochromatin requires a class of sRNAs called primal RNAs (priRNA; Marasovic *et al.*, 2013). Similar to piRNAs, priRNAs are formed from longer Ago-bound ssRNAs by a 3′-5′ exonuclease, called Triman.

No true miRNA has so far been described from fungi. Instead, *Neurospora crassa* expresses a class of analogous sRNAs that, similar to miRNAs, derive from precisely processed hairpin stems (Lee *et al.*, 2010). These miRNA-like RNAs (milRNA) have a strong preference for 5′ U, as has been noted for miRNAs in many eukaryotic species (Avesson *et al.*, 2012; Tarver *et al.*, 2015). Unlike miRNAs, milRNAs are however generated through distinct biogenesis mechanisms, some of which are completely Dcl-independent (Lee *et al.*, 2010). Some milRNAs need Dcl to generate the precursor, but not he mature milRNA. Yet others depend on Dcl for both the pri-milRNA and pre-milRNA cleavage steps, similar to plant miRNAs. Ago-bound milRNA-1 is formed by exonuclease 3′-5′ trimming (Xue *et al.*, 2012), which resembles the maturation process of animal piRNAs and yeast priRNAs by the action of Trimmer and Triman, respectively.

tRNAs are present in all cellular life forms and constitute a ubiquitously expressed potential sRNA precursor. Indeed, tRNA-derived RNA fragments (tRFs) have been discovered in many bacterial, archaeal and eukaryotic species (Gebetsberger and Polacek, 2013; Kumar *et al.*, 2014). This relatively recently described sRNA class might thus be evolutionary very old. Possibly, tRFs constitute an ancient form of regulatory sRNA. The tRF biogenesis mechanisms are in many cases obscure, but tRNA anticodon loop cleavage enzymes have been identified in a few organisms. These proteins belong to diverse ribonuclease families: PrrC and Colicins in bacteria, RNase A in animals and RNase T2 in yeast (Megel *et al.*, 2015). Accumulating evidence indicates that many tRFs are functional regulatory sRNAs. Common to a range of organisms is induced tRF production under stress conditions and a role for tRFs is in control of translation initiation or elongation (Ivanov *et al.*, 2011; Gebetsberger *et al.*, 2012; Sobala and Hutvagner, 2013).

Ago immunoprecipitation (Ago IP) and sRNA deep sequencing have shown that interaction between tRFs and Ago proteins is conserved between many organisms. In human cell lines, a number of tRFs were identified that associate primarily with Ago1 (Burroughs *et al.*, 2011). A different study revealed that the B-cell expressed tRF CU1276 associate with all four human Agos. CU1276 acts as a miRNA and suppresses the proliferation rate in lymphoma cells by Ago-dependent posttranscriptional repression (Maute *et al.*, 2013). Analysis of photoactivatable-ribonucleoside-enhanced crosslinking and

immunoprecipitation (PAR-CLIP) data showed that tRFs and miRNAs are produced by different biogenesis mechanisms, but that both interact strongly with Ago around sRNA nt 9-13. Also similar to miRNAs, the 5′ ends of many of the analyzed tRFs match predicted miRNA seed regions, suggesting that tRFs interact with target mRNAs through seed-pairing (Kumar *et al.*, 2014). In Ago-IP deep sequencing from *A. thaliana*, tRFs were found in the libraries from four out of six examined Ago proteins (Loss-Morais *et al.*, 2013). The human parasite *Trypanosoma cruzi* does not employ RNAi, but its genome encodes a Piwi-class Ago protein. Electron microscopy showed the *T. cruzi* Piwi to colocalize with tRFs in intra- and extracellular vesicles. In addition, uptake of these vesicles by host cells was observed (Garcia-Silva *et al.*, 2014).

1.3 *Phytophthora infestans*

1.3.1 An introduction to oomycetes

Phytophthora infestans belongs to the class oomycota. These filamentous eukaryotic microorganisms were previously classified as fungi, but are now recognized as members of the kingdom Stramenopila (Figure 4). Consequently, oomycetes are sister species to diatoms and brown algae, and are more closely related to Alveolata and Rhizaria than to Fungi (Adl *et al.*, 2012; Burki, 2014; Thines, 2014). The earliest fossil evidence of oomycetes is from the Devonian, 416-359 million years ago (mya; Krings *et al.*, 2011) and molecular clock estimations place the emergence of oomycetes in the mid-Paleozoic, 430-400 mya (Matari and Blair, 2014). The identification of oomycete structures inside fossilized plant tissue indicates that association with plant cells was an early feature of members of this lineage. The absence of evidence of disease in the plant fossils suggests that the first oomycetes were saprotrophs rather than pathogens (Krings *et al.*, 2011). Studies of extant early diverging oomycetes however point toward a parasitic lifestyle of the last common oomycete ancestor. Since the majority of basal species are obligate parasites with marine or freshwater hosts, the oomycete origin was probably in the sea. The ancestor species might have been brought onto the land by nematode hosts, or by doing a host jump from seaweed to early coastal plants (Beakes *et al.*, 2012; Fawke *et al.*, 2015).

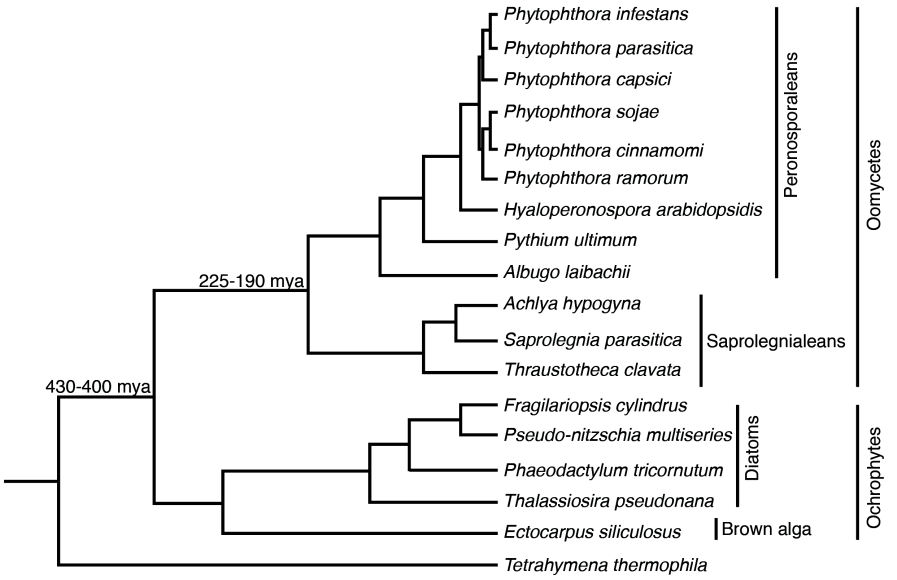

Figure 4. Phylogenetic relationships and divergence times between Stramenopiles. Molecular clock methods calibrated with fossil data were used to estimate the time of divergence between oomycetes and diatoms/brown algae to 430-400 mya. Saprolegnialeans and peronosporaleans split approximately 225-190 mya. The ciliate *Tetrahymena thermophila* was used as outgroup. The tree and divergence time data were adapted from Matari and Blair (2014).

A number of phenotypic characteristics distinguish oomycetes from fungi. The cell walls of fungi typically contain glucans, chitin and glycoproteins, but no cellulose (Bowman and Free, 2006). Oomycete cell walls are composed of cellulosic and non-cellulosic glucans. GlcNAc, the monomeric unit of chitin, was detected at over five percent in the cell wall of *Aphanomyces euteiches*, but *Phytophthora* cell walls lack chitin (Melida *et al.*, 2013). In contrast to fungi, the hyphal structures of oomycetes rarely have septa, which makes the hypha multinucleate (coencytic) and tube-like (Judelson and Blanco, 2005). Likewise, the asexual spores of oomycetes (called sporangia) are multinucleate, whereas fungal spores contain one nucleus per cell. The motile spores of oomycetes (the zoospores) are biflagellate, while fungal spores have no, or only one, flagellum (Thines, 2014). The oomycetes and fungi are thus classified as bikonts and unikonts, respectively. Both fungi and oomycetes secrete hormones to induce the sexual stage of their life cycles (Judelson and Blanco, 2005). In contrast to their fungal counterparts, oomycete mating hormones are however not peptide-based. The *Phytophthora* mating hormones have been identified as terpenes (Ojika *et al.*, 2011).

With exception for the sexual structures, oomycetes are diploid. This is another difference to fungi, whose nuclei are predominantly haploid (Judelson

and Blanco, 2005). Since some lineages have experienced expansions of repetitive DNA during the course of evolution, oomycete genomes vary in size (Raffaele and Kamoun, 2012). To date, *Albugo laibachii* (37 Mb) and *P. mirabilis* (280 Mb) have the smallest and largest fully sequenced oomycete genomes, respectively (Raffaele *et al.*, 2010; Kemen *et al.*, 2011). Members of *Phytophthora* clade 1c, where *P. mirabilis* and *P. infestans* belong, have highly expanded genomes (Raffaele *et al.*, 2010) and the *P. infestans* reference genome is 240 Mb (Haas *et al.*, 2009). TEs and repeats constitute 74% of the *P. infestans* genome, the most highly proliferated sequence element of which is *Gypsy* long terminal repeat (LTR) retrotransposons (Haas *et al.*, 2009; Raffaele *et al.*, 2010). The *P. infestans* genome stands out also in comparison to Stramenopiles outside of the oomycete lineage: the diatom *Phaeodactylum tricornutum* has a genomic DNA of 27 Mb (Bowler *et al.*, 2008) and the genome of the human parasite *Blastocystis* sp. is merely 18.8 Mb (Denoeud *et al.*, 2011).

The life strategies of oomycetes are diverse. Some are free-living saprophytes while others are pathogenic (Thines, 2014). The plant pathogenic species can be further classified as biotrophs, hemibiotrophs and necrotrophs. Briefly, biotrophic bacterial, fungal or oomycete pathogens exploit living plant tissue to acquire nutrients and to propagate. Some eukaryotic biotrophs and hemibiotrophs develop intracellular hyphae or haustoria, through which nutrients are obtained and/or molecules are secreted. Necrotrophs, on the other hand, need to kill the host tissue in order to feed on it. *P. infestans* is a hemibiotroph, and as such, starts the infection process as a biotroph, proceeding into necrotrophy at a later stage (Latijnhouwers *et al.*, 2003; Fawke *et al.*, 2015).

To date, the genus *Phytophthora* (greek for "plant destroyer") contains over 100 species, a number that is increasing as new species are continuously discovered (Kroon *et al.*, 2012). More than 60% of all known oomycetes are plant pathogenic and *Phytophthora* species cause some of the most serious plant diseases worldwide (Raffaele and Kamoun, 2012). Other plant-infecting oomycetes include the downy mildews (obligate biotrophs such as *Hyaloperonospora arabidopsidis*), Pythiales (e.g. the necrotroph *Pythium ultimum*, and saprotrophs), Albuginales (obligate biotroph white rusts, e.g. *Albugo candida*) and Saprolegniales (e.g. *Saprolegnia parasitica*; Judelson, 2012; Fawke *et al.*, 2015).

1.3.2 Potato late blight

P. infestans has a quite narrow host range, infecting mainly solanaceous plants, of which the interactions with potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.) are the most well-studied. The disease caused by *P. infestans* is called late blight and is the major infectious disease affecting potato (Haverkort *et al.*, 2009). According to estimations, the annual global cost of agricultural control efforts and potato tuber yield loss (16%) was around €5 billion in 2009 (Haverkort *et al.*, 2009). Nevertheless, many people are more aware of late blight for historical reasons. In the 1840's, a severe outbreak of late blight hit Western Europe. The disease particularly affected Ireland, where the Great Famine caused mass-emigration and the death of around one million people (Turner, 2005). The Irish population, heavily dependent on potato for their daily calorie intake, was decimated by around 20% between the years 1845 and 1851. Herbarium samples from potato and tomato leaves collected at the time of the Great Famine have lately been subjected to genomic DNA sequencing (Yoshida *et al.*, 2013). Mapping of sequence reads to the *P. infestans* reference genome showed that the 1840's late blight outbreak was caused by a single *P. infestans* strain, which was a sister lineage, and not a direct ancestor, of the strain that dominated throughout the world during the 20th century.

The *P. infestans* disease cycle (Figure 5) is initiated by germinated cysts or germinated sporangia (Erwin and Ribeiro, 1996; Fry, 2008). These propagules are able to infect potato stem, tuber, or leaf tissue. During the first days after infection, no disease symptoms are visible, a distinctive feature of a biotrophic interaction. After around two days, infested foliage typically starts to yellow and to show small spots of necrosis. Next, sporangiophores are formed on the abaxial side of the leaf and sporangia are released. Sporangia can spread by wind or rain to nearby plants or distant fields and initiate new infections. At temperatures of around 10-15°C, sporangial zoospore release is triggered. Zoospores are biflagellated and motile cyst precursors. Their encystment and germ tube formation start new disease cycles. Under conditions that are optimal for the pathogen (cool and moist), the disease cycle takes no more than four days. Both sporangia and zoospores can infect tubers when dispersed to the soil by water or wind. Diseased tubers typically show brown or purple spots on their skin before starting to rotten (Erwin and Ribeiro, 1996; Fry, 2008).

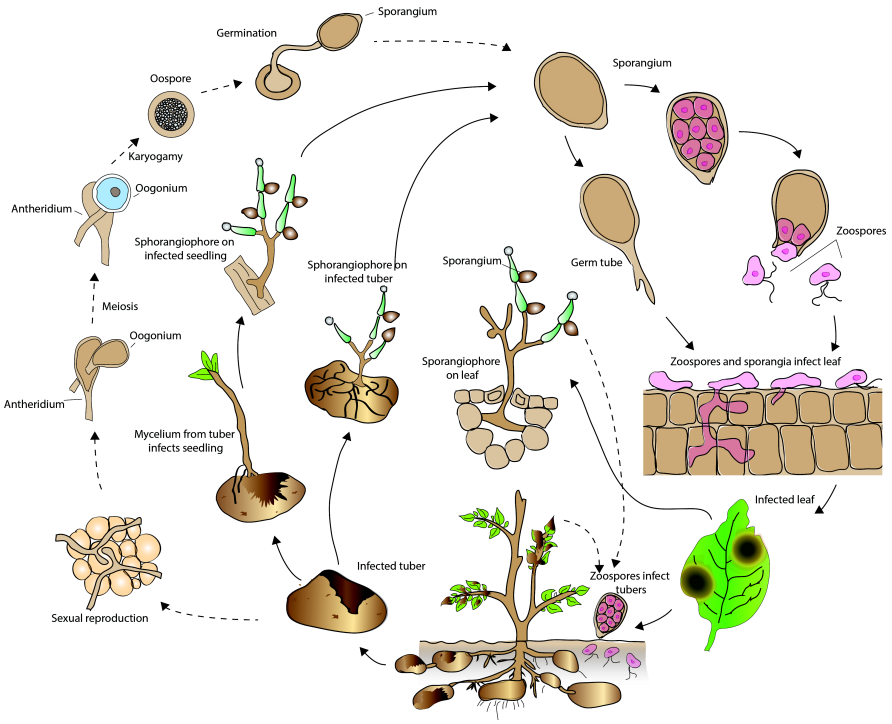

Figure 5. Life cycle of *P. infestans*. The different stages are described in the main text. Adapted from Agrios (2005) and Fry (2008).

Late blight control has traditionally been focused on fungicide applications and on growing potato cultivars bred for *P. infestans* resistance (Haverkort *et al.*, 2009). Resistance breeding is based on potato crossings with wild *Solanum* relatives carrying late blight resistance (*R*) genes (described in chapter 1.5; (Vleeshouwers *et al.*, 2011). Chemical sprayings are a necessity to protect potato fields from virulent *P. infestans* strains. Consequently, large amounts of fungicides are used every year in areas where potatoes are grown, leading to increased costs for the grower (chemicals, fuel, manpower), and to augmented CO_2 emissions (Jones *et al.*, 2014). In addition, the effects of fungicides on other organisms in the ecosystem are largely unknown. Blight forecasting can help to eliminate unnecessary agrochemical use and to reduce the risk of pathogen resistance development (Small *et al.*, 2015). The behavior of the pathogen is however hard to predict, especially under varying weather conditions. Neither *R* gene-based breeding nor fungicide applications have proven durable as control strategies, as *P. infestans* notoriously has evolved both the ability to counter-attack plant resistance genes and to overcome various fungicides (Haverkort *et al.*, 2009; Jones *et al.*, 2014).

One percent of the agricultural area on Sweden was used for potato cultivation in 2014. In comparison, the two major crops wheat and barley covered 18% and 13% of the agricultural land, respectively (Jordbruksverket, 2015a; Jordbruksverket, 2015b). Still, due to the destructive effect of *P. infestans*, a large proportion of all applied fungicides is applied on potato fields in Sweden. In contrast to many other European countries, where clonal lineages of *P. infestans* normally dominate (Cooke *et al.*, 2012), both mating types are prevalent (Sjoholm *et al.*, 2013). The reason for this difference has not been firmly established, but climatic factors are probably involved. The hardy oospores that are formed through mating are able to survive cold winters outside of the host, in contrast to asexual mycelia that depend on living plant tissue (tubers) to overwinter (Andersson *et al.*, 2009).

1.4 Potato

1.4.1 Potato as a food crop

In terms of human consumption, potato is the world's third most important food crop after wheat and rice (Haverkort *et al.*, 2009). The crop is cultivated in almost all parts of the world. While potato production is declining in the developed world, it is gaining in importance in the developing countries. The global potato production was 376 million tons (Mt) in 2013 (FAOSTAT, 2015). The largest potato producers were China (96 Mt), followed by India (45 Mt), the Russian Federation (30 Mt), Ukraine (22 Mt) and USA (20 Mt).

Potato was domesticated between 6,000-10,000 years ago in the Andean highlands of Peru and Bolivia (Spooner, 2006). The first report of potato in Europe was from 1567 in the Canary Islands (Ames and Spooner, 2008). The early European potatoes probably originated from landraces in the high Andes, while modern potato cultivars predominately originate from lowland Chile.

S. tuberosum has an autotetraploid chromosome complement and is highly heterozygous. These two features have complicated the generation of a high-quality genomic sequence from potato. To facilitate genome assembly and physical mapping, a double monoploid potato clone (DM) was used to obtain the genomic reference sequence (Potato Genome Sequencing Consortium *et al.*, 2011). The haploid potato genome, distributed over 12 chromosomes, turned out to be 844 Mb and was predicted to contain 39,031 genes.

Potato tubers are starch-rich and contain as much protein as cereals (Rodriguez-Falcon *et al.*, 2006). They form from swelling underground stolons under short-day, low-temperature conditions. Freshly formed tubers undergo a period of dormancy before they start sprouting. Tuber-producing potato relatives are only found within the *Solanum* section *Petota* (Spooner, 2006).

Identification of genes involved in the tuberization process will benefit the breeding for traits such as tuber dormancy and the ability to tuberize under long-day conditions. Whole transcriptome sequencing of DM and a heterozygous diploid potato line identified over 300 genes upregulated during stolon-to-tuber transition (Potato Genome Sequencing Consortium *et al.*, 2011). Three classes of genes were strongly upregulated: (1) patatin-encoding genes, (2) genes involved in starch biosynthesis and (3) genes coding for Kunitz protease inhibitors. The protein products of the latter are associated with pathogen defense.

1.4.2 Potato sRNAs

Potato sRNAs are 21-24 nt long, with 24 nt as the major sRNA size class (Zhang *et al.*, 2013; Lakhotia *et al.*, 2014). This is similar to *A. thaliana* and rice, where sRNA sequencing showed that the 24 nt peak correspond to repeat-associated siRNAs (see e.g. Kasschau *et al.*, 2007; Morin *et al.*, 2008). The second largest size class in potato is 21 nt, which is the characteristic size of miRNAs and trans-acting siRNAs in *A. thaliana* (Kasschau *et al.*, 2007). Several potato miRNAs belong to conserved plant miRNA families (Zhang *et al.*, 2013; Lakhotia *et al.*, 2014). In some of the miRNA families, only one family member has been identified from potato, while the largest families contain 6-12 individual members (e.g. the miR156, miR171 and miR399 families). Most of the identified potato miRNAs originate from intergenic regions and those expressed from genic regions are mainly intronic. A recent study described the discovery of 147 potato-specific miRNAs by deep sequencing (Lakhotia *et al.*, 2014). While the majority of these novel miRNAs were represented by low sequence read counts, reads from a corresponding miRNA* were identified for all candidates. The presence of a miRNA* is a sign of pre-miRNA cleavage by Dcl and distinguishes true miRNAs from other classes of sRNAs. The detection of a miRNA* is therefore a critical criterion for miRNA annotation (Kozomara and Griffiths-Jones, 2014).

1.5 Oomycete effectors and potato R proteins

Plant defense against pathogen infection is based on innate immunity (Jones and Dangl, 2006). This defense system is described as two-layered and employs two major classes of immune receptors. The first line of defense consists of external transmembrane pattern recognition receptors (PRRs), which identify conserved and essential pathogen molecules (pathogen associated molecular patterns, PAMPs), such as fungal chitin or bacterial flagellin (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Known oomycete

PAMPs include the peptide Pep-13 from *P. sojae*, small sterol carrier proteins (such as *P. infestans* Inf1), and the cellulose-binding elicitor lectin CBEL from *P. parasitica* (Zipfel, 2014; Fawke *et al.*, 2015). Recently, the receptor-like protein ELR was identified as the host protein mediating recognition of *P. infestans* Inf1 (Du *et al.*, 2015). Many pathogens are prevented from intrusion by PRR-mediated recognition (PAMP-triggered immunity; PTI). Yet, adapted pathogens have evolved effector proteins, which give them the ability to overcome PTI.

The second layer of plant immunity acts through intracellular receptors (R proteins), specialized in detecting pathogen effectors. Direct or indirect effector recognition activates host defense responses that lead to effector triggered immunity (ETI). Detected effectors are called avirulence (Avr) proteins (Jones and Dangl, 2006). ETI often involves a type of localized cell death termed the hypersensitive response, which stops the pathogen from establishment and spreading in the plant tissue. The R protein-induced defense response is typically stronger and faster than PTI (Jones and Dangl, 2006). Typical R proteins have nucleotide binding (NB) and leucine-rich repeat (LRR) domains and are classified as either TNL or CNL receptors, based on their N-terminal domain identities (Marone *et al.*, 2013). TNLs have N-terminal TIR domains, which share sequence similarity with the *Drosophila melanogaster* Toll and human interleukin-1 receptors, while CNLs have coiled–coil (CC) domains. The haploid potato genome contains 755 *R* genes, of which the majority (584 genes) code for CNLs (Jupe *et al.*, 2013).

To date, 68 *Solanum* late blight *R* genes have been identified, some of which have been used in potato breeding programs (Rodewald and Trognitz, 2013). Classical resistance breeding is however time-consuming and complex, due to crossing barriers, linkage drag and the tetraploid, highly heterozygotic potato genome (Vleeshouwers *et al.*, 2011; Rodewald and Trognitz, 2013). The speed of *R* gene introduction can be significantly increased by genetic engineering techniques. Potato crops carrying so called "cisgenic" modifications are produced by introducing *R* genes from crossable potato relatives and excluding exogenous selection markers (Jacobsen, 2013). This is in contrast to transgenic organisms, which carry genes or regulatory sequences derived from species outside of their natural gene pool.

Plant pathogens employ effector proteins in a multitude of ways in order to interfere with host defense responses (de Jonge *et al.*, 2011). Effectors are diverse in structure, function and delivery mechanisms. Some are targeted to the host apoplasm, where they facilitate host tissue penetration or target plant extracellular defenses. Examples of apoplastic effectors are cell-wall-degrading enzymes and inhibitors of plant proteases and glucanases (Kamoun, 2006; de

Jonge *et al.*, 2011). Other effectors are translocated into the plant cell where they interact with host intracellular proteins. *P. infestans* expresses two large classes of cytoplasmic effectors: Crinklers (CRNs) and RxLRs. Their point of delivery into the host cell is most likely via the haustorium (Petre and Kamoun, 2014). CRN and RxLR N-termini are conserved within the two protein families and specify secretion and targeting to the host cytoplasm. Their highly variable C-termini mediate the biochemical activity inside host cells (Bos *et al.*, 2006; Raffaele and Kamoun, 2012). The *P. infestans* reference genome comprises 196 *CRN* genes, 255 *CRN* pseudogenes and 563 *RxLR* genes. The name CRN stems from the ability of this group of proteins to induce cell death when expressed in plant cells ("crinkling and necrosis"). RxLRs are named after the short amino acid motif present in the N-termini of all members of this family (arginine, any amino acid, leucine, arginine). All known *P. infestans* Avr proteins are RxLRs. CRN and RxLR-encoding genes are located in gene-sparse regions of the genome, where transposon density is high (Haas *et al.*, 2009). The positioning of *CRN* and *RxLR* genes close to mobile genetic elements is believed to facilitate expansion of these two large gene families and most likely underlies the high evolutionary rates of *P. infestans* effectors.

A subset of intracellular effectors targets the host nucleus, where they interact with nuclear host proteins and interfere with processes such as transcription and RNA processing (Rovenich *et al.*, 2014). Some of the CRNs in *P. infestans* and *P. capsici* localize to the host nucleus (Schornack *et al.*, 2010; Stam *et al.*, 2013). PiCRN8, which has demonstrated kinase activity, requires nuclear localization to induce host cell death (van Damme *et al.*, 2012). A study of fluorescently tagged RxLR effectors from *H. arabidopsidis* revealed host nuclear localization to be common: out of 49 tested RxLRs, 33% targeted the host nucleus, while another 33% localized to both the nucleus and the cytoplasm (Caillaud *et al.*, 2012). Membrane-localization was observed for 26% of the effectors. Screening for *P. sojae* effectors that suppress host RNAi identified a nuclear-localized RxLR that interferes with host Dcl1 subnuclear localization (Qiao *et al.*, 2015; also see chapter 1.6.3). An example of bacterial host nucleus translocated effectors is the *Xanthomonas* transcription activator-like (TAL) effectors, which, as the name implies, act as activating transcription factors (Boch *et al.*, 2009).

1.6 Small RNA in plant-pathogen interactions

1.6.1 Host sRNAs employed in defense

Besides the well-studied antiviral RNAi response, plants also use RNA silencing to protect themselves against bacterial and eukaryotic pathogens. Two siRNAs have been implicated in antibacterial immunity in *A. thaliana*: the 40 nt long siRNA lsiRNA-1 and the natural antisense siRNA (nat-siRNA) ATGB2 (Katiyar-Agarwal *et al.*, 2006; Katiyar-Agarwal *et al.*, 2007). Both siRNAs are induced upon bacterial infection and target negative regulators of disease resistance.

miRNAs have been identified as important components of the plant immune system. Specific miRNA families are involved in the regulation of *R* genes encoding CNL and TNL proteins (Park and Shin, 2015). By suppressing *R* gene expression in the absence of pathogen infection, this system ensures that a high level of R protein is produced only when the plant is attacked by a pathogen. This likely limits the fitness cost of multi-copy R protein expression and prevents autoimmunity. In *A. thaliana*, the miRNA regulatory circuit is amplified by secondary siRNAs formed from *R* gene transcripts subsequent to miR472-directed cleavage. This pathway represses genes encoding CNL-type receptors and regulates both PTI and ETI (Boccara *et al.*, 2014).

The genomes of solanaceous plants contain large numbers of genes coding for TNL and CNL immune receptors, and miRNA-mediated *R* gene regulation seems to be a conserved feature in this plant family. Specifically, *Nicotiana tabacum* miR6019 and miR6020 play an important role in defense against tobacco mosaic virus by regulating the transcript levels of the *N* immune receptor (Li *et al.*, 2012). The same study also identified three potato miRNA families that target *R* genes and validated their corresponding mRNAs as miRNA targets. In tomato, miRNAs of the miR482/miR2118 superfamily directs cleavage of CNL-encoding transcripts (Shivaprasad *et al.*, 2012). Silencing of these CNLs is relieved upon infection by viruses or a bacterium that encodes RNA silencing suppressors. This observation raises the possibility that the mechanism of pathogen-inducible *R* gene expression has evolved to counterattack pathogen-expressed silencing suppressors. Recently, downregulation of the miR482/miR2118 superfamily was reported from *P. sojae*-infected soybean (Zhao *et al.*, 2015). Predicted soybean targets of miR482/miR2118 and secondary siRNAs include a large number of TNLs and CNLs.

Additional information about specific plant sRNAs involved in defense is described by Pelaez and Sanchez (2013).

1.6.2 RNA-directed DNA methylation in plant defense

Two plant-specific polymerases, RNA polymerase IV and V, are dedicated to RdDM. In the canonical pathway in *A. thaliana*, 24 nt siRNAs are produced by Dcl3 from RNA polymerase IV transcripts that have been converted to dsRNA by Rdr2 (Matzke *et al.*, 2015). The 24 nt siRNAs guide Ago4 to nascent RNA polymerase V transcripts, marking loci to become targeted by *de novo* DNA methylation. Silencing can also spread to the chromatin level, by induction of repressive chromatin marks such as methylation of histone 3 lysine 9 (H3K9me). Transcriptional silencing by RdDM plays many important roles in *A. thaliana*. In addition to TE repression, it is involved in processes such as imprinting, female germ cell specification and pathogen defense (Matzke and Mosher, 2014). Studies of plants carrying mutated RdDM components have shown the critical role of this pathway in defense against DNA viruses (Pumplin and Voinnet, 2013). The circular geminivirus genome, which associates with host histones as a part of the infection process, is silenced by both DNA methylation and H3K9me (Raja *et al.*, 2008). In accordance with the important role of RdDM in plant defense, viruses have evolved RNA silencing suppressors that target this pathway (Hamera *et al.*, 2012). Heterochromatic siRNAs and methylation are involved also in defense against bacterial infections (Yu *et al.*, 2013). The promoter of the *R* gene *Resistance methylated gene 1* (*Rmg1*) in *A. thaliana* contains two helitron TE insertions, which are hotspots for RdDM. *Rmg1* silencing is relieved under bacterial infection and in RdDM mutants.

RdDM is a plant-specific gene regulatory process. Studies of the evolution of the core RdDM proteins and the occurrence of 24 nt sRNAs in plants and *Chlamydomonas reinhardtii* suggests that RdDM was present already in the first land plants (Matzke and Mosher, 2014; Huang *et al.*, 2015). Repeat-associated 24 nt sRNAs are found at high levels in monocots and dicots, and in reproductive tissues in gymnosperms. They are however expressed at low levels in moss (Cho *et al.*, 2008) and absent from *C. reinhardtii* (Molnar et al., 2007). The latter organism also lacks RNA polymerases IV and V, and the RdDM-specific RNAi components Rdr2, Dcl3 and Ago4 (Matzke and Mosher, 2014).

The *P. infestans* genome has a repeat content of 74% and does not encode a homolog of DNA methyltransferase (Haas *et al.*, 2009). The genome of the oomycete *Pythium ultimum*, on the other hand, has 7% repeats and codes for DNA methylases (Levesque *et al.*, 2010). The link between TE expansion and DNA methylation in oomycetes remains to be examined.

1.6.3 Pathogen-controlled RNA silencing in virulence

To counteract plant sRNA-based defenses, pathogens express molecules that suppress the host RNA silencing system. The first viral suppressors of RNA silencing (VSRs) were discovered in 1998 (Pumplin and Voinnet, 2013). VSRs counteract plant RNAi in various ways, e.g. by sequestering viral siRNAs or by inhibiting antiviral factors such as Ago1 and Rdr6. That bacteria would have evolved silencing suppressors was anticipated for many years, and in 2008, three *Pseudomonas* effectors with silencing suppressing activity were identified (Navarro *et al.*, 2008). AvrPtoB, AvrPto and HopT1 interfere with three different steps of the miRNA pathway: pri-miRNA transcription, pre-miRNA processing and Ago1 function, respectively.

Eukaryotic silencing suppressors have so far only been identified in oomycetes. Through a screening of *P. sojae* effectors that interfere with plant RNA silencing, two RxLRs (designated PSR1 and PSR2) were identified that reduce the levels of sRNAs in the host (Qiao *et al.*, 2013). PSR1 perturbs the levels of both miRNAs and siRNAs, but PSR2 has a more specific effect, affecting only secondary siRNAs. PSR1 localizes to the host nucleus, where it interacts with a positive regulator of *A. thaliana* immunity. By doing so, it interferes with pri-miRNA processing by Dcl1 (Qiao *et al.*, 2015). Importantly, both effectors increase the virulence of potato virus X and *P. infestans* in *Nicotiana benthamiana*, showing that RNA silencing, and its suppression, play important roles in *Phytophthora* pathogenicity.

A vast number of proteinaceous effectors have been characterized from fungi, bacteria and oomycetes. Given the intimate contact that pathogens establish with host plant cells, they should have ample opportunities to translocate "effector" molecules other than proteins. sRNAs, due to their low molecular weight, simple base pairing rules and universal presence, are good candidate virulence factors. The necrotrophic pathogen *Botrytis cinerea* secretes such sRNA effectors into the host, and some of them have been shown to downregulate host immunity genes under *B. cinerea* infection (Weiberg *et al.*, 2013). The fungal-derived sRNAs have the same size as plant endogenous sRNAs, are processed by fungal Dcl proteins and bind host Ago1.

sRNAs also move in the other direction, from the host into the pathogen (Koch and Kogel, 2014). Host-induced gene silencing (HIGS) is a strategy that has been used experimentally to silence pathogen genes by expression of sRNA precursors in host plant cells. HIGS has proven successful against oomycete, fungal, insect and nematode pathogens, and parasitic plants. Currently, very little is known about the mechanism of sRNA transport between plants and their pathogens. Likewise, whether plants translocate endogenous sRNAs to target pathogen transcripts has not been resolved.

2 Aims of the present study

The gains from increased knowledge of RNA-guided biological processes are many. Put in a larger perspective, increased understanding of RNA function will enable methodological advances, which will favor plant research and science in general. The focus of this thesis has been on sRNAs in the plant pathogen *P. infestans*. The general aim was to increase our understanding of the gene regulatory systems in this organism, knowledge that is key to understanding concepts such as evolution of pathogenicity and suppression of transposon activity. The study also aimed to examine how pathogens and hosts exploit sRNAs as part of their infection and defense strategies. Finally, my hope was that the work would give new insights into the diversity of sRNA silencing systems in eukaryotes.

Specifically, the original goals of my PhD studies, and the defined objectives added as the projects proceeded, were to:

- Reveal which classes of sRNAs are expressed by *P. infestans*,
- Examine to what extent effector genes are regulated by sRNAs,
- Determine whether *P. infestans* Argonaute (PiAgo) proteins have redundant or specialized roles in sRNA-directed silencing, and what specific functions each PiAgo protein has,
- Identify the sRNA classes that are produced from transposable elements,
- Characterize sRNAs derived from tRNA
- Explore the possibility of using the CRISPR/Cas9 method to study gene function in *P. infestans*, and
- Develop a method to silence pathogen genes by the expression sRNA-generating constructs in the host plant.

3 Results and Discussion

3.1 Interspecies sRNA transport (Paper IV)

HIGS is a method to silence pathogen genes by the expression of double-stranded RNA in a host plant. By the design of silencing constructs that are complementary to genes present only in the intended organism, this technique holds promise of being highly pathogen-specific (Koch and Kogel, 2014). Since sRNA-directed silencing is known to work efficiently in *P. infestans* (Whisson *et al.*, 2005), we wanted to develop and test HIGS in the *P. infestans*-potato pathosystem. If successful, HIGS could work as a multi-purpose tool, to (i) assess pathogen genes important for infection, (ii) generate plants with enhanced pathogen defense, (iii) study potential sRNA transport between host and pathogen and (iv) dissect the factors needed for such transport to occur.

3.1.1 A new tool to study *P. infestans*-potato interactions

Three *P. infestans* genes, whose products had previously proven to play important roles in the pathogen during infection, were chosen for evaluation of the HIGS method: *PiGpb1*, *PiCesA2* and *PiPec* (Latijnhouwers and Govers, 2003; Grenville-Briggs *et al.*, 2008; Ospina-Giraldo *et al.*, 2010). The effect of targeting *PiGapdh*, whose protein product functions in energy metabolism (Sirover, 1999), was also studied. Transgenic potato lines expressing hairpin (hp) constructs targeting the four genes were generated, and the disease phenotypes were assessed after *P. infestans* inoculation. The largest disease reduction was observed for plants expressing a hp complementary to the gene coding for PiGpb1 (G-protein beta-subunit 1), which is needed for sporangia formation (Latijnhouwers and Govers, 2003). The amount of *P. infestans* biomass decreased steadily in the hp-Gpb1 plants relative to the wild type plants as the infection proceeded from 24 to 48 and 72 hours post inoculation (hpi). This was accompanied by a relative decrease in the *PiGpb1* transcript levels in the same transgenic lines. While the wild type plants were clearly

diseased at 30 days after inoculation, the hp-expressing potato plants showed much less pronounced symptoms.

The silencing constructs employed a constitutive plant promoter, and quantitative reverse transcription PCR (qRT-PCR) confirmed the expression of all four constructs in the corresponding transgenic lines. Nevertheless, the levels of sRNAs homologous to *PiGpb1* in the hp-PiGpb1 plants were very low, and detectable only at 24 hpi by Northern hybridization (25-28 nt sRNAs). A similar result was obtained by Illumina sRNA sequencing from *P. infestans*-infected hp-PiGpb1 plants: the read counts from *PiGpb1* (24, 25 nt long) were at background levels at all three assayed time points. Despite these observations, sRNAs were most likely generated, since: (i) the disease symptoms were evidently reduced in the hp-PiGpb1 potato plants compared to controls, (ii) expression of hpRNA could be demonstrated in the hp-PiGpb1 lines, and (iii) sRNAs are known to be efficiently produced in hp-expressing potato plants (Missiou *et al.*, 2004). Sampling before 24 hpi or after 72 hpi would perhaps allow sRNA detection. A significant decrease in *PiGpb1* transcript abundance was observable at 48 hpi, which indicates that host-generated sRNAs were present in the pathogen before this time point, and that silencing needs to build up to a certain threshold before being fully active in the pathogen.

3.1.2 Mechanistic aspects of plant-pathogen sRNA transfer

The mechanism behind pathogen-plant sRNA transfer is currently unknown. mRNA and sRNA can spread cell-to-cell (through plasmodesmata) and systemically (through phloem) in plants (Sarkies and Miska, 2014), but how the HIGS signal is transferred into pathogen tissue remains to be shown. Other intriguing questions concern to what extent endogenous sRNAs also move from host cells into their pathogens, whether sRNA transport is equally frequent in both the pathogen-to-host and the host-to-pathogen directions, and which functions such endogenous translocated sRNAs would have.

Vesicular transport is an important means of intercellular communication in eukaryotic cells and a good candidate mechanism for interspecies sRNA transport (Knip *et al.*, 2014). Indeed, mammalian exosomes contain functional mRNAs and miRNAs (Valadi *et al.*, 2007; Kim *et al.*, 2014). Exosomes were suggested as the transport vehicle in the *Blumeria graminis*-barley HIGS system (Nowara *et al.*, 2010), and these vesicles have been observed to accumulate at the *B. graminis*-host interface. At the contact point between *P. infestans* and *N. benthamiana*, endosomes were observed to surround haustoria by live-cell imaging (Lu *et al.*, 2012). Moreover, vacuole-targeted endosomes were seen to re-localize to the extrahaustorial membrane during infection

(Bozkurt *et al.*, 2015). Lipid receptor-mediated endocytosis has been proposed as the delivery mechanism of oomycete proteinaceous effectors into plant cells, but more experiments are needed to finally confirm that this is the case (Tyler *et al.*, 2013; Wawra *et al.*, 2013).

To study the sRNA transport process and to determine which host and pathogen factors that are required for interspecies RNA silencing, new experimental techniques are needed. In light of this, HIGS could be used to screen for *P. infestans* protein factors needed for sRNA uptake, by knocking down candidate genes and assessing the effect on HIGS efficiency. The method could also be applied to dissect which pathogen RNA silencing factors that mediate HIGS, by targeting e.g. *PiRdr1* and *PiAgo1-5*.

3.1.3 Potato miRNA regulation during infection (unpublished)

As of October 2015, miRBase21 listed 224 pre-miRNAs and 343 mature miRNA sequences from potato (SolTub3.0; http://www.mirbase.org). This number is expected to increase as more studies of potato sRNAs are carried out. Investigating endogenous sRNA pathways in potato will be critical to our understanding of the *P. infestans*-potato pathosystem, since miRNAs play important roles in plant defense (chapter 1.6.1) and pathogens de-regulate host miRNA signaling pathways during infection (chapter 1.6.3). To analyze how potato miRNAs respond to *P. infestans*, we are currently conducting transcriptome and sRNA sequencing studies on *P. infestans*-infected potato samples. The objective of the project is to identify differentially regulated potato miRNAs and their target mRNAs, and to validate target gene product function by experimental approaches. Potentially, this will reveal new gene regulatory circuits active during pathogen infection and disentangle some of the molecular mechanisms used by *P. infestans* to counter-attack the host immune system.

3.2 sRNAs in *P. infestans*

When this PhD project started, the identities of the sRNA populations in *Phytophthora* were unknown, and the sRNA-interacting proteins had not been characterized. RNAi was however used as a tool to analyze gene function in *P. infestans*, and genes encoding homologs of key silencing factors had been predicted from the newly sequenced *P. infestans* genome: four Agos (PiAgo1/3/4/5), one RdRP (PiRdr1) and one Dcl (PiDcl1; Vetukuri *et al.*, 2011a). *PiAgo2* is a gene duplicate of *PiAgo1*; the two genes share 99% nucleotide sequence identity and are located in close proximity in the genome. Figure 6 depicts the domain architecture of *P. infestans* silencing proteins.

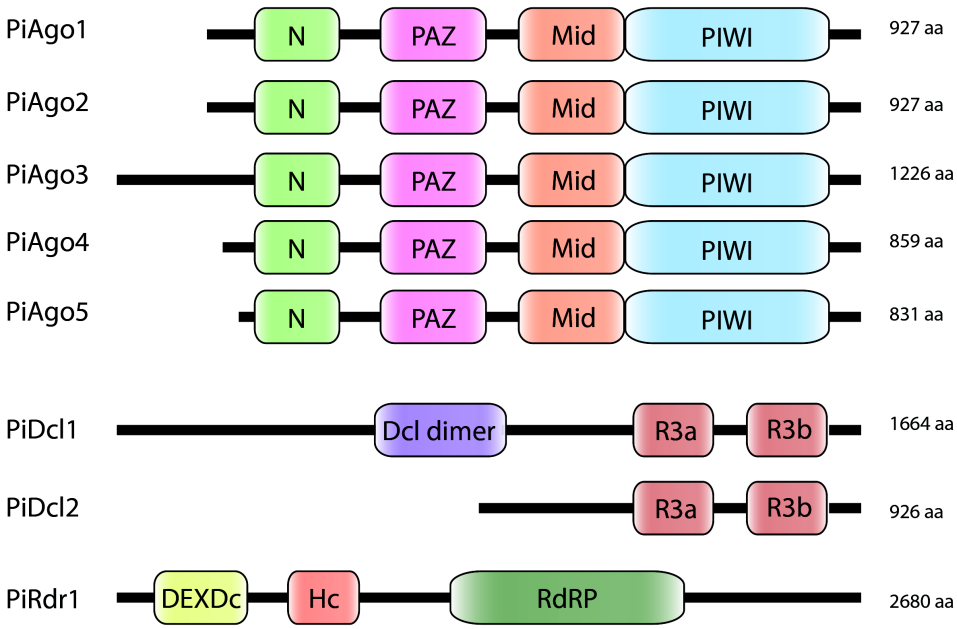

Figure 6. Domain architecture of *P. infestans* Ago, Dcl and RdRP proteins predicted by SMART (Letunic *et al.*, 2015). Individual proteins are not drawn to scale. N, amino-terminal; PAZ, Piwi-Argonaute-Zwille; Mid, middle; PIWI, P element-induced wimpy testis; Dcl dimer, Dicer dimerization; R3a and R3b, RNase III; DEXDc, DEAD-like helicase; Hc, helicase C-terminal; RdRP, RNA-dependent RNA polymerase.

The majority of the *P. infestans* genome is composed of TEs and repeats (Haas *et al.*, 2009). Transposon movement can cause deleterious mutations, gene expression alterations or chromosomal rearrangements (Girard and Hannon, 2008). Uncontrolled activity of *P. infestans* repetitive elements would therefore have severely negative effects on the organism. The mechanism of TE control in *P. infestans* is not known, but seems to involve sRNA-directed heterochromatin formation (van West *et al.*, 2008). The genome lacks genes encoding DNA methyltransferases, but is predicted to encode a number of chromatin regulatory proteins (Vetukuri *et al.*, 2011a). Moreover, *P. infestans* effector genes are typically located in close proximity to TEs, and transposon silencing likely contributes to effector gene expression regulation (Whisson *et al.*, 2012). For these reasons, one of the goals of this project was to characterize sRNAs derived from effector genes and TEs.

To study the sRNA pathways in *P. infestans*, three main experimental approaches were used: sRNA sequencing, stable gene silencing and protein-RNA co-immunoprecipitation (co-IP). Our initial sequencing analyses were

performed on the isolates R0 and 3928A, whereof the first is weakly pathogenic on potato and the latter is highly aggressive (Flier *et al.*, 2001; Cooke *et al.*, 2012). A number of sRNA-generating loci were also studied in *PiDcl1* and *PiAgo* silenced lines (Paper I). The subsequent project focused on sRNAs derived from tRNA in R0 and 3928A, and included samples sequenced from three infection-stage time points in isolate 88069 (Paper II). Finally, the roles of the PiAgo proteins were analyzed through co-IP and deep sequencing (Paper III).

3.2.1 sRNA characteristics (Paper I, II)

Mapping of sRNA sequence reads generated from R0 and 3928A to the *P. infestans* genome revealed two major sRNA size classes, centered on 21 and 25 nt (Paper I). This finding is in line with the identification of a second *PiDcl* gene (*PiDcl2*) in NCBI's sequence trace archive (Fahlgren *et al.*, 2013). An additional class of sRNAs was found by analysis of reads mapping to tRNA; tRNA-derived RNA fragments (tRFs). These were 19-40 nt in length and had a bias towards 5′ G (Paper II).

Examination of the 5′ and 3′ ends of sRNAs is informative of their biogenesis pathways. For example, products of RNase III enzymes (e.g. Drosha and Dcl) have 3′ overhanging ends, a 5′ monophosphate and a 3′ hydroxyl group (Court *et al.*, 2013). Secondary siRNAs in *C. elegans* on the other hand, are directly generated by RdRP and have di- or triphosphorylated 5′ ends (Ghildiyal and Zamore, 2009). To gain insights into the sRNA pathways in *P. infestans*, we analyzed the sRNA termini by enzymatic and chemical assays (Paper I). Terminator exonuclease degrades RNA with a 5′ monophosphate, but is inactive on RNA having a 5′ triphosphate or a modified 5′ end. Total sRNAs from *P. infestans* were treated with the Terminator enzyme and the 5′ end structure of sRNAs from *Copia* LTR3 and *Crypton6* were assayed by Northern hybridization. sRNAs generated from the two transposons were sensitive to the treatment, suggesting 5′ monophosphorylated ends. Likewise, treatment with Tobacco acid pyrophosphatase (TAP), which converts 5′-triphosphosphate or 5′-capped ends into 5′ monophosphate ends, had no effect. The combination of Terminator and TAP however stimulated sRNA degradation. The conclusion from this experiment is that sRNAs from *Copia* LTR3 and *Crypton6* most likely are generated through PiDcl processing.

Methylation on the 2′-*O* of the 3′ terminal ribose serves as a protective mechanism against the action of sRNA 3′ uridylation and 3′-5′ exonuclease-dependent degradation (Ji and Chen, 2012). Treatment of sRNAs with sodium periodate followed by β-elimination leads to removal of the 3′ nt in sRNAs that have unmodified 3′ ends, while 3′ end-methylated sRNAs are protected

from β-elimination (Tang and Zamore, 2004). Northern hybridization showed a migratory shift for *Copia* LTR3 sRNAs after β-elimination, which demonstrates that the sRNA 3´ ends are non-methylated. This is in line with the absence of a homolog of the sRNA methyltransferase *Hen1* in the *P. infestans* genome.

3.2.2 sRNAs from TEs and effector genes (Paper I)

As expected from the repeat-rich constitution of the *P. infestans* genome, the majority of sequenced sRNAs from R0 and 3928A derived from transposons, with LTR retroelements being the largest source. The majority of TE-mapping sRNAs were 21, 25 and 26 nt long, but sRNAs detected from *Crypton6* and *Gypsy-Pi1* by Northern hybridization were longer, 32 and 35 nt, respectively. This difference might be attributed to the limited read length of the SOLiD sequencing technique, which at the time this project was carried out allowed analysis of up to 33 nt long reads.

sRNAs from *CRN* effector genes were centered on 21 nt, whereas sRNAs from *RxLRs* had a bimodal size distribution, similar to sRNAs generated from the whole genome. To analyze whether effector gene expression might be regulated by sRNAs during interaction with the host plant, candidate *RxLRs* and *CRNs* were evaluated by qRT-PCR using RNA samples extracted from *P. infestans*-infected potato leaves. In many cases, an inverse relationship was observed between the presence of sRNAs and the accumulation of particular target effector transcripts in the two isolates. This suggests that sRNA-directed processes control *RxLR* and *CRN* genes in *P. infestans*.

Furthermore, we identified mRNA and sRNA expression differences between the pathogenic (3928A) and non-pathogenic (R0) isolate (Flier *et al.*, 2001; Cooke *et al.*, 2012) that correlated with their respective infection phenotypes on the host plant. For example, 30 and 35 nt sRNAs from *PiAvr3a* were detectable in R0, which did not express this gene. sRNAs were barely detectable in 3928A, which expressed *PiAvr3a* throughout infection. The situation was reversed in the case of the two *CRNs* PITG_18133 and PITG_22969, where very low transcript levels in 3928A were accompanied by easily detectable 21 nt sRNAs. The *in planta* expression levels of these two genes were much higher in R0, especially at 24 hpi, and no sRNAs were observed in this isolate.

A similar correlation between the presence of sRNAs and the expression of an *RxLR* gene is seen at the *PsAvr3a* locus in *P. sojae* (Qutob *et al.*, 2013). In this study, the levels of 24-26 nt long sRNAs were significantly higher in silenced lines than in those where the *PsAvr3a* transcript was detectable. Evidence is emerging that sRNA-directed effector gene silencing is a common

way of pathogens to modulate their virulence and adapt to new hosts (Gijzen *et al.*, 2014; Weiberg *et al.*, 2014). Further studies are needed to clarify the mechanisms behind the observations described above. For example, what factors control the timing of sRNA expression? Which chromatin alterations accompany target gene repression? And which signals are needed to induce the reversal of silencing?

3.2.3 tRNA-derived RNA fragments (Paper II)

A common step in sRNA sequence data analysis is the removal of reads from abundant ncRNAs, such as tRNA and rRNA degradation products. Recent characterization of the tRNA-mapping read fraction has however established tRFs as a genuine class of sRNAs (Megel *et al.*, 2015). Contrary to what is expected from non-systematic tRNA degradation, tRFs have highly homogenous 5′ and 3′ ends, and their levels do generally not correlate with the expression level or gene copy number of the parental tRNAs (Gebetsberger and Polacek, 2013).

In R0 and 3928A, about 2% of the sRNA sequence reads mapped to tRNA. The majority of all tRFs in the different life cycle stages derived from the 5′ part of the tRNA (5′ tRFs). A similar bias toward 5′ tRFs was seen in sequenced infection stage samples from 88069, where the majority of tRFs corresponded to tRNA half-molecules (29-33 nt). The presence of 5′ tRNA halves was validated by Northern hybridization. Strong signals from 34 nt long 5′ fragments were detected, and consistent with tRNA cleavage in the anticodon loop, the detected 3′ halves were typically 40 nt.

All tRNAs were not equally potent tRF producers: a fragment from $tRNA^{Ile}$ (Ile0-5′tRF) accounted for up to 25% of all tRFs. The overrepresentation of Ile0-5′tRF did not correlate with the number of $tRNA^{Ile}$ genes, and pointed toward an as yet unknown role played by this tRF in *P. infestans* biology. Analysis of sRNA sequencing libraries generated from *P. infestans*-infected potato leaves revealed reduced levels of 32 nt Ile0-5′tRFs during infection. On the contrary, many other tRFs increased in abundance during infection. Some of these accumulated differentially at the three sampled time points, possibly representing sRNAs with regulatory roles during the progression from biotrophic to necrotrophic growth.

The tRNA cleavage enzyme in *P. infestans* has not been identified. From studies conducted in yeast, *Tetrahymena thermophila* and mammalian cells, it is known that some tRFs depend on Dcl and Ago proteins (Haussecker *et al.*, 2010), while other tRFs are produced through distinct pathways. In yeast and mammals, tRNA halves are generated by RNase T2 and RNase A enzymes, respectively (Thompson and Parker, 2009; Yamasaki *et al.*, 2009). RNase T2 is

a potential tRF biogenesis factor in *P. infestans*, since five homologs are predicted from the genomic sequence.

To investigate the involvement of *P. infestans* RNA silencing factors in the tRF pathways, we generated transgenic lines expressing hairpin silencing constructs targeting, individually, *PiDcl1* and the *PiAgos*. Probing for tRFs by Northern hybridization, a clear reduction in the levels of specific tRFs was observable in the *PiAgo1* silenced line. This indicates that PiAgo1 is needed for tRF production or stability. It is possible that *P. infestans* tRFs function as siRNA-like guides to induce PiAgo-mediated RNA silencing. Alternatively, they play regulatory roles by competing with other endogenous sRNAs for PiAgo binding. *PiDcl2* had not yet been identified at the time when we generated the *PiDcl1* and *PiAgo* silenced lines. The involvement of PiDcl2 in the tRF pathway therefore remains to be examined.

3.3 *P. infestans* Dcl and Ago proteins

3.3.1 PiDcl and PiAgo dependencies of sRNAs (Paper I, II)

Oomycete sRNA pathways are understudied in comparison to homologous systems in plants and animals. A number of protein factors involved in RNA silencing have been identified in mammalian, fly, worm and plant model organisms (reviewed by Ghildiyal and Zamore, 2009; Meister, 2013; Bologna and Voinnet, 2014). As a first step toward functional characterization of the sRNA machinery in *P. infestans*, we chose to study the core RNAi factors Dcl and Ago.

To assay the sRNA dependencies on *P. infestans* RNAi factors, levels of individual sRNAs were analyzed by Northern hybridization in transgenic *PiDcl1* and *PiAgo* silenced lines. Clearly reduced accumulation of 21 nt sRNAs from a *Copia* LTR transposon and a *CRN* gene was seen in the *PiDcl1* knockdown transformant. Northern hybridization provided no evidence for PiDcl1-dependence of tRFs. Nor did we observe any significant differences on global tRF levels in the sRNA sequencing datasets generated from the *PiDcl1* silenced line and the wild type. Longer sRNAs (32 nt) from the effector-encoding gene *PiAvrblb1* were negatively affected by depletion of *PiAgo4* and *PiAgo5*. Taken together, the results from Northern hybridization and sRNA sequencing indicate involvement of PiDcl1 in the 21 nt sRNA pathway, while PiAgo1 seems to interact with tRFs and PiAgo4 and PiAgo5 appear to associate with long sRNAs. The sRNA dependency on PiAgo3 is still obscure, since attempts of silencing *PiAgo3* were unsuccessful.

With two major sRNA size classes and two PiDcl proteins, it is reasonable to assume that PiDcl1 and PiDcl2 generate one size class each. The anticipated role of PiDcl2 in the 25 nt sRNA pathway needs to be tested. Specific recruitment of a particular PiDcl (likely PiDcl1) to *CRN* transcripts is suggested from the 21 nt size of *CRN*-derived sRNAs. The mechanism behind this association is another question for the future to answer. Finally, it has yet to be established whether individual PiAgos are functionally coupled to specific PiDcl proteins and sRNA types, as in *D. melanogaster* and *A. thaliana* (Okamura *et al.*, 2004; Creasey *et al.*, 2014). Another possibility is that the PiAgos act redundantly, as do human Agos in the miRNA pathway (Burroughs *et al.*, 2011).

3.3.2 Genome-wide analysis of Ago-bound sRNAs (Paper III)

sRNA Northern blot is a rather low-throughput method, where one genomic locus is assayed at a time. To analyze the sRNA binding properties of *P. infestans* Ago proteins on a larger scale, we used PiAgo-sRNA co-IP and deep sequencing.

A first indication that the different PiAgo proteins interact with separate classes of sRNAs was obtained through end-labeling of co-IP sRNA with ^{32}P and denaturing gel electrophoresis. Specifically, PiAgo1 and PiAgo4 bound the two major sRNA size classes: 21 nt and 25 nt, respectively. The sizes of deep sequenced sRNAs agreed with the initial gel analysis: the PiAgo1 IP was highly enriched for 20-22 nt sRNAs, while sRNAs in the PiAgo4 IP were mainly 24-26 nt long. The PiAgo3 sample showed no specific sRNA size-class enrichment, indicating that the IP from PiAgo3 was unsuccessful. PiAgo5 was enriched for 21 nt sRNAs, but not to the same extent as PiAgo1.

In plants, the identity of the 5′ terminal nt dictates sorting of sRNAs into different Ago complexes (Mi *et al.*, 2008). Inspection of the 5′ ends of the PiAgo IP sRNAs revealed PiAgo4 to be enriched for sRNAs bearing 5′ U and PiAgo1 to prefer sRNAs with 5′ C. Analysis of TE-derived sRNAs discovered additional differences between the PiAgo1 and PiAgo4 IP samples. The majority of sRNAs from *Gypsy* LTR elements and *Mutator* and *helENtron* DNA transposons were 20-21 nt and associated with PiAgo1. In contrast, *Copia* LTR, and *Helitron, Crypton* and *PiggyBac* DNA elements accumulated 24-26 nt sRNAs, which were proportionately more abundant in the PiAgo4 sample than in the control. Consistent with their 21 nt size (Paper I), sRNAs from *CRN* genes were highly abundant in the PiAgo1 sample. *RxLR*-derived sRNAs were few in numbers and were not enriched in any PiAgo co-IP.

TGS and PTGS act mainly in the nucleus and cytoplasm, respectively (Castel and Martienssen, 2013). To reveal the subcellular localizations of

PiAgo1 and PiAgo4, GFP-tagged versions of the two proteins were studied by confocal microscopy. Both proteins were found to be present in the cytoplasm, which indicates roles in post-transcriptional control. The samples studied were from *in vitro* grown sporulating mycelium, and it is possible that one or both proteins translocate to the nucleus under other growth conditions or in different life cycle stages.

In conclusion, the global analysis of Ago-interacting sRNAs in *P. infestans* indicates that sorting of sRNAs is dictated both by sRNA size and 5′ nt identity. The data suggests that PiAgo1 and its bound 20-22 nt sRNAs regulate *CRN* gene expression and control the activity of *Gypsy, Mutator* and *helENtron* transposons. Other TE classes appear to be controlled by the dual action of PiAgo1/20-22 nt sRNAs and PiAgo4/24-26 nt sRNAs. Taken together with the results from our knockdown experiments, PiAgo1 and PiDcl1 seem to be connected through the 21 nt sRNA pathway.

A number of intriguing questions remain to be answered. For example, how do the PiAgo-sRNA association patterns look like under infection? Do *CRN*-associated 20-22 nt sRNAs contribute to variation in pathogenicity between different *P. infestans* strains? What TE features determine their processing into either 21 or 25 nt sRNAs? PiAgo5 was linked to both 21 nt sRNAs and long sRNAs. How can these two findings be reconciled?

3.3.3 Does *P. infestans* have miRNA? (Papers I, III)

Using the sRNA datasets from R0 and 3928A, six candidate miRNAs were predicted bioinformatically. These 21-24 nt long candidates were present in both isolates and showed no homology to any miRNA from any other organism deposited in miRBase (www.mirbase.org). The putative miRNAs were however rather weak candidates, since their respective miRNA* sequences could not be detected in the sequencing libraries. A single miRNA (miR8788) has since then been described in oomycetes (Fahlgren *et al.*, 2013). This miRNA is conserved between *P. infestans*, *P. sojae* and *P. ramorum* and reads corresponding to a miRNA* were detectable in all three species.

In a second attempt to identify miRNAs in *P. infestans*, we used the PiAgo co-IP sRNA datasets and the miRNA prediction tool ShortStack. Since this strategy would potentially enrich for functional PiAgo-bound sRNAs, the chances of finding miRNAs were expected to increase compared to the previous search. One good miRNA candidate, identical to miR8788, was predicted. Northern hybridization with PiAgo co-IP RNA samples detected miR8788 specifically in the PiAgo1 IP sample.

miRNAs, as we know them from well-studied model organisms, are generated by clearly distinct biogenesis pathways, separated from those of siRNAs and piRNAs. Moreover, the target silencing mechanism sometimes differs between miRNAs and other sRNAs, and miRNAs frequently have their own dedicated protein interaction partners. To evolve a miRNA machinery for the production of a single miRNA thus seems unlikely, and additional miRNAs might await discovery in *P. infestans*. Possibly, miRNAs in this organism are too divergent from canonical miRNAs to be detected by common prediction algorithms. A distinct possibility is that an ancestral oomycete possessed a higher number of miRNAs and that they are progressively being lost in evolutionary time. In this perspective, three studies of miRNAs in non-oomycete Stramenopiles are informative. While the brown alga *Ectocarpus siliculosus* apparently expresses a large number of miRNAs (Tarver *et al.*, 2015), no good miRNA candidates have been found in the diatoms *Thalassiosira pseudonana*, *Fragilariopsis cylindtrus* and *Phaeodactylum tricornutom* (Lopez-Gomollon *et al.*, 2014; Rogato *et al.*, 2014). The latter organism instead produces a class of miRNA-like sRNAs that shows the Dcl signature 2-nt 3′ overhang on only one side of the miRNA/miRNA* duplex.

3.4 Adapting the CRISPR/Cas9 technology to *P. infestans* (unpublished)

Gene knockout in *Phytophthora* species has proven challenging, most likely due to diploidy and low rates of homologous recombination (Judelson, 1997). Studies of gene function have therefore employed methods for transient or stable gene silencing, which in most cases lead to incomplete knockdown of gene expression (Whisson *et al.*, 2005; Ah-Fong *et al.*, 2008). In addition, silenced transformants in *P. infestans* sometimes revert back to wild type gene expression levels after some time of subculturing (Vetukuri *et al.*, 2011b).

In order to enable gene disruption, we aimed at adapting the CRISPR/Cas9 technology for use in *Phytophthora* species. To enable expression, nuclear localization and correct folding of the sgRNA, a suitable ncRNA promoter first needed to be identified. The most commonly employed sgRNA promoter is the RNA polymerase III promoter driving expression of U6 spliceosomal RNA (Ran *et al.*, 2013; Ranganathan *et al.*, 2014). According to the Rfam annotation of the sequenced reference genome, *P. infestans* is predicted to have 250 U6 genes (www.broadinstitute.org). A candidate U6 promoter was chosen, based on 5′-RACE verification of transcriptional activity and transcript starting nucleotide. The promoter, sgRNA and FLAG-tagged Cas9 were cloned into a single oomycete expression vector. For the initial assessment of the method,

PiCdc14 was selected for targeted gene disruption. Knockdown of this gene had previously shown to drastically reduce the number of formed spores, an easily scored phenotype (Ah Fong and Judelson, 2003).

Transformation of the sgRNA-Cas9 construct into *P. infestans* generated five transformants, but none of them showed reduced sporulation. Cas9 was detected by Western blot in three of the transgenic lines, two of which also expressed sgRNA (Figure 7). Possibly, the absence of a clear phenotype in these two transformants was attributable to Cas9 or sgRNA mislocalization, as the two components need to be co-expressed in the same nucleus for DNA cleavage to take place. Future studies should aim at examining nuclear targeting, by for example fluorescence microscopy or subcellular fractionation, and at optimizing the sgRNA-Cas9 vector for efficient gene mutation.

Successful employment of the CRISPR/Cas9 technology in *P. infestans* is likely achievable in the near future, since the system was very recently proven functional in *P. sojae* (Fang and Tyler, 2015).

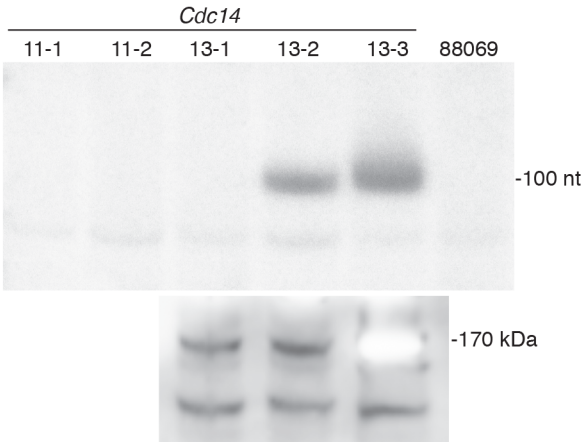

Figure 7. sgRNA and Cas9 detection in transgenic *P. infestans* lines. Upper panel: Northern hybridization detection of sgRNA in two out of five transformants. 20 nt *PiCdc14* guide sequence oligo plus 82 nt sgRNA scaffold form a 102 nt sgRNA. Lower panel: Western blot detection of Cas9 protein (163 kDa) in three transformants (13-1, 13-2 and 13-3). The numbers 11 and 13 designate different *PiCdc14* guide sequence oligos. 88069: wild type.

4 Conclusions

The following are the most interesting conclusions from this work.

- The main sRNA classes in *P. infestans* center on 21 and 25 nt and are PiDcl products.
- Silencing of *PiDcl1* abolishes the production of particular 21 nt sRNAs.
- sRNAs contribute to the regulation of *CRN* and *RxLR* effector genes.
- tRFs represent a class of highly expressed sRNAs in *P. infestans*. Most tRFs are generated through cleavage in the tRNA anticodon loop area. The levels of certain tRFs are affected by depletion of *PiAgo1*.
- Our phylogenetic analysis indicates the presence of two oomycete Ago clades: one PiAgo1-like and one PiAgo3/4/5-like.
- Expression of protein-coding genes is likely regulated by a pathway involving PiAgo1, PiAgo5 and 20-22 nt sRNAs.
- Transposon silencing seems to be mediated through two pathways; one involving 20-22 nt sRNAs and one acting through PiAgo4 and 24-26 nt sRNAs.
- PiAgo1 has roles in both suppression of transposon activity and in regulation of protein-coding genes. PiAgo1 binds *pin*-miR8788 and a large number of *CRN*-derived sRNAs.
- Expression of hairpin silencing constructs in potato enables downregulation of endogenous *P. infestans* genes. This method, HIGS, holds promise to work as a tool to study gene function in *P. infestans*. It could also be used as a means to generate potato with increased late blight defense.

5 Future perspectives

The results from this thesis work suggest a number of additional experiments to perform and pinpoint challenges for future studies of RNA silencing processes in *P. infestans.*

Considering the many different kinds of sRNAs identified in organisms such as plants, fungi and ciliates, additional sRNA classes are likely to be discovered in *Phytophthora*. What roles might such undiscovered sRNAs play? It would also be of interest to examine to what extent the sRNA pathways are shared between different *Phytophthora* species.

Additional studies are needed to firmly establish the individual roles of PiDcl1 and PiDcl2 in the biogenesis of 21 and 25 nt sRNAs. A long-term goal would be to determine the structural basis for the generation of two distinct sRNA sizes by PiDcl1 and PiDcl2.

Northern hybridization detected 32 nt long sRNAs from *Avrblb1*. This is a non-canonical sRNA size in *P. infestans*, and it would be interesting to further study the proposed involvement of PiAgo4 and PiAgo5 in biogenesis of long sRNAs.

Since no transformant lines silenced for *PiAgo3* or expressing PiAgo3-GFP were obtained, this gene is so far uncharacterized. The size, 5´ nt and genomic origin of PiAgo3-preffered sRNAs are important missing aspects of the sRNA landscape in *P. infestans*.

Regarding tRFs, future studies could focus on assessing the levels of these sRNAs under stress conditions, and to further probe their PiAgo requirements. In addition, the role of PiDcl2 in this pathway has yet to be tested, and so is the

involvement of RNase T2 enzymes. The role of tRFs could be studied by using reporter gene constructs carrying target sites for specific tRFs or by inhibition of tRF expression through introduction of tRF-complementary oligonucleotides.

The contribution of individual PiDcl and PiAgo proteins to PTGS and TGS needs to be determined. Confocal microscopy suggested cytoplasmic localization of PiAgo1 and PiAgo4, which indicates roles in PTGS. Colocalization with marker proteins could potentially reveal the exact cytoplasmic compartments these two proteins localize to. Likewise, studying the subcellular distribution of PiAgo3 and PiAgo5 will show whether one of these proteins is targeted to the nucleus. The identity of any additional factors involved in TGS respective PTGS and which specific chromatin alterations that mediate transcriptional silencing are two additional issues to resolve.

HIGS holds great promise as a tool to study gene function and to engineer potato with enhanced defense against *P. infestans*. Upcoming studies could test to simultaneously silence multiple *P. infestans* genes. The technique could also be used to study the mechanism of host-pathogen RNA transport, by targeting of candidate *P. infestans* genes needed for RNA uptake.

It will be important to improve the tools for functional genomics in *Phytophthora* species. In view of this, adopting the CRISPR/Cas9 system to *P. infestans* should have high priority. Hopefully, it will be possible in the near future to disrupt *PiAgo*, *PiDcl* and *PiRdr* genes, and to assess their functions through sRNA sequencing from mutant lines. Similarly, by generation of active-site mutant proteins, CRISPR/Cas9 could be used to test whether a catalytic triad is required for PiAgo slicer activity.

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