Small RNAs in *Phytophthora infestans* and cross-talk with potato

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Cover: Thematic illustration of host induced gene silencing in potato-*P. infestans* pathosystem.
(photo: Sultana Nilufar Jahan)

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Abstract

Small RNAs (sRNAs) are small non-coding RNAs usually ranging in size 20-30 nt. They are playing important roles in plant-pathogen interactions. This thesis aimed at studying the sRNA populations in potato and *Phytophthora infestans* and their role in the potato-*P. infestans* interaction. An attempt was also made to implement such knowledge to improve resistance in potato against *P. infestans.*

P. infestans is an oomycete that causes severe damage to potato and tomato, and is well known for its ability to evolve rapidly to overcome resistance. It possesses active RNA silencing pathways and sRNAs are playing important roles to control the large numbers of transposable elements (TE) present in its genome and effector genes. Through deep sequencing of sRNAs from two isolates of *P. infestans* differing in pathogenicity, three clear size classes (21, 25/26 and 32 nt) of sRNAs were identified. RxLR and Crinkler (CRN) effector gene-derived sRNAs were present in both isolates, but exhibited marked differences in abundance. Some effector genes, such as PiAvr3a and PiAvrblb2, to which sRNAs were found, also exhibited differences in transcript accumulation between the two isolates. Majority of sRNAs also mapped to TEs. An additional group of sRNAs, the tRNA-derived RNA fragments (tRFs) ranging in size from 19-40 nt was identified in *P. infestans* as well. Some tRFs accumulated differentially during infection.

A host-induced gene-silencing (HIGS) approach was proven to be successful in the potato-*P. infestans* pathosystem. Four different endogenous genes in *P. infestans* were targeted in my study by HIGS constructs and choice of target gene was shown crucial for a successful outcome. HIGS has the potential to be adopted in new resistance breeding to improve resistance in potato against *P. infestans*.

Single late blight resistant potato cultivar was sequenced upon infection with compatible isolate of *P. infestans*. In order to decipher the molecular events underlying its resistance breakdown, analysis of transcript change and the possible role of sRNAs on transcript regulation during infection are ongoing. In infected potato, resistance genes are targeted by its own miRNAs leading to suppression. Additional components are most likely involved in this process and near future analysis will help to enhance our understanding of how *P. infestans* uses sRNAs to evade plant immune responses.

Keywords: effector, HIGS, miRNA, *Phytophthora infestans,* potato, sRNA

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Dedication

To my beloved family (my parents, sister, husband and daughter)

Imagination is more important than knowledge.
Albert Einstein

Contents

List of Publications

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

I Vetukuri RR, Åsman AKM, Tellgren-Roth C, **Jahan SN**, Reimegård 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 **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. 66:2785-2794

IV **Jahan SN,** Åsman AKM, Fogelqvist J, Dixelius C. Can *Phytophthora infestans* modulate host miRNA to evade defense responses? (manuscript).

Additional publications

Vetukuri RR, Åsman AKM, **Jahan SN**, Avrova AO, Whisson SC, Dixelius C. 2013. Phenotypic diversification by gene silencing in *Phytophthora* plant pathogens. Comm. & Integrative Biol. 6:e25890.

The contribution of Sultana N. Jahan to the papers included in this thesis was as follows:

I Shared the laboratory work with Ramesh Vetukuri and Anna Åsman. Performed cloning, RNA extraction, Northern blots, *Phytophthora* transformations.

II Participated in design of the project, generated sRNA sequencing data for potato - *P. infestans* interaction. Participated in data analysis.

III Participated in design of the project along with main supervisor and co-workers, performed most of the laboratory works, analyzed the data and wrote the paper in cooperation with co-authors.

IV Designed the project together with main supervisor and co-workers, participated in the analysis of bioinformatics data, performed most of the laboratory work, analyzed the data and wrote the manuscript in collaboration with co-authors.

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
NB-LRR	Nucleotide binding site, leucine-rich repeat
ncRNA	Non-coding RNA
PAMP/MAMPs	Pathogen or microbe triggered molecular pattern
PTGS	Post-transcriptional gene silencing
RdRP	RNA-dependent RNApolymerase
RNAi	RNA interference
RxLR	Arginine any-amino-acid leucine arginine
siRNA	Small interfering RNA
sRNA	SmallRNA
TE	Transposable element
TGS	Transcriptional gene silencing
TNL	Toll interleukin-1 receptor, nucleotide binding site, leucine-rich repeat
tRNA	Transfer RNA
tRFs	tRNA-derived RNA fragments

1 Introduction

World food security is under threat due to intense pressure of the world population growth, climate change, the waning of biodiversity and newly emerging plant and animal diseases. To this end reducing crop losses is of immense importance. Worth mentioning in this perspective is the Irish potato famine in the 1840s caused by *P. infestans* which led to the death toll of about one million people (Nusteling, 2009). In order to control infectious diseases in crops plant breeders have been exploiting resistance (*R*) genes for decades, with varying degrees of success. The rapid pace of pathogen evolution is a major obstacle for the maintenance of sustainable and durable disease resistance. The main challenge is the blending of different forms of resistance traits to provide long-lasting resistance. The research today with increasing genomic and genetic understanding of host–pathogen interactions contribute to such knowledge.

In this summary, I will give a brief overview on the *Solanaceae* plant family, the oomycete pathogen *P. infestans* and the small RNAs. In order to cover this wide range of topics mostly recent review papers have been cited.

1.1 *Solanaceae,* an important plant family

Solanaceae is a large plant family belonging to the order *Solanales*, comprising about 100 genera and 2500 species (Olmstead *et al.*, 2008). The largest genus is *Solanum* with approximately 1500 species (Weese and Bohs, 2007). *Solanaceae* plants can be of different forms like, herbs, shrubs, trees, vines, lianas and also epiphytes. They are important source of food, spices and as ornamentals and even as medicine. Well-known representatives are potato, tomato, eggplant, tobacco, petunia and pepper. Considering the economic importance, *Solanaceae* is the third most important plant taxon and most important in terms of vegetable crops. *Solanaceae* plants normally contain high levels of alkaloids some of which have pharmacological and medical importance, like the tropane alkaloid in belladonna and datura (Prance, 1972; William, 1979). There are also toxic glycoalkaloids and calystegine alkaloids in some members in this plant family including the important crop species potato, tomato, and eggplant (Valkonen *et al.*, 1996; Friedman and McDonald, 1997; Biastoff and Drağer, 2007). In cultivated potato the major glycoalkaloids are α-chaconine and α-solanin, which can be poisonous. The safety limit of glycoalkaloid consumption is 200 mg total glycoalkaloid/kg fresh weight of unpeeled raw potato (Jadhav *et al.*, 1981).

1.1.1 Potato an important member in the *Solanaceous* plant family

After wheat and rice, potato is the third most important food crop, with a worldwide production of 376 million tons (2013, http://faostat.fao.org/). Its importance as a crop is also increasing in the developing countries (Mullins *et al.*, 2006). Tubers are important dietary source of starch, protein, antioxidants and vitamins (Camire *et al.,* 2009). Other than being food, potato starch has a wide variety of commercial uses ranging from food additives and pharmaceutical excipients to ingredient in paper and clothing industry (Kraak, 1993). Despite the importance of the potato, the genetics and inheritance of many important qualitative and quantitative agronomic traits is not well

understood. This is mainly due to the tetraploid nature of the genome (2n=4x=48), the high degree of heterozygosity and the absence of homozygous inbred lines and the lack of a collection of genetically well-defined marker stocks. A range of ploidy levels exist and several wild *Solanum* species do not set tubers.

Different *Solanaceae* species are sexually separated by different ploidy levels and endosperm balance numbers (EBN) (Panahandeh *et al.*, 2008). For a successful cross, both parents should have the same EBN. The problem of ploidy level difference and EBN variation could partly be overcome by using a haploid inducer e.g. *S. phureja* pollinations (Rokka, 2003). This approach can be utilized to increase genetic diversity and transfer valuable traits like disease resistance genes (Rokka, 2009). Details on various improvements of the potato crop can be found in the following reviews (Simko, 2004; Ortiz *et al.*, 2009; Watanabe, 2015).

1.1.2 *Solanaceae* genomes

The phylogenetic relationship and estimated divergence time of selected *Solanaceae* species is presented in Fig. 1. Accordingly, potato and tomato are closely related compared to the other species. The most important *Solanaceous* crops: potato, tomato, tobacco and pepper are now sequenced (Table 1). These four species have different morphologies, but genome analysis has disclosed high similarities in terms of gene content and organization. Analyzing the conserved syntenic segments (CSS) in these species revealed 17 distinct and well-conserved segments (Wang *et al.*, 2008). There are some small-scale gene duplications and losses. The tomato genome shows 8% sequence diversity from potato genome involving nine large and several smaller inversions (Sato *et al.*, 2012). The genome sequences have revealed gene family expansion,

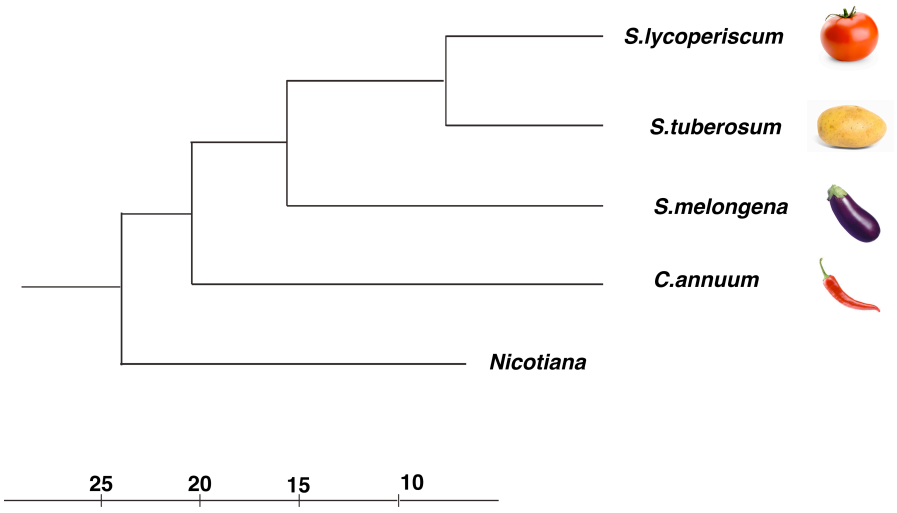

Figure 1. Phylogenetic tree of selected *Solanacae* species and estimated divergence time. The scale bar represents the estimated times for divergence between species in million years. *Nicotiana* represents an extinct species, the most recent common ancestor of *N. tomentosiformis* and *N. acuminate*. Adapted from Wu and Tanksley (2010).

tissue specific gene expression and recruitment of genes to new pathways contributing to tuber or fruit formation in different genera of this plant family

Table 1. *Genome data of important Solanaceae species.*

Species	**Genome size**	**Gene models**	**References**
Potato *(Solannum tuberosum)*	844 Mb	39,031	(Xu *et al.*, 2011)
Tomato *(Solanum lycoperiscum)*	900 Mb	34,727	(Sato *et al.*, 2012)
Tobacco *(Nicotiana tabacum)*	4.5 Gb	93,000	(Sierro *et al.*, 2014)
Hot pepper *(Capsicum annum)*	3.48 Gb	34,903	(Kim *et al.*, 2014)

(Kim *et al.*, 2014). Analysis of *R* genes encoding nucleotide-binding site (NBS) shows that in the hot pepper genome, the expansion of these genes resulted in loss of collinearity with NBS-coding regions in potato and tomato. But higher synteny is maintained between potato and tomato NBS-coding regions.

1.2 The late blight disease

The late blight pathogen *Phytophthora infestans* is native to central Mexico, and reported for the first time 1843 in the USA (Grunwald and Flier, 2005). The literal meaning of *P. infestans* is "plant destroyer", a name given by Anton de Bary in 1861, as he proved that this organism caused late blight to potato. It possess highly epidemic potential, infecting leaves, stems and tubers and the whole plant can be destroyed in few days (Fry, 2008). The Irish potato famine in the mid-1840s was the starting point of recognition of the late blight disease and its pathogen *P. infestans* including new resistance breeding attempts. The late blight disease is still the major cause of loss in potato and tomato production (Nowicki *et al.*, 2012). Worldwide, an economic loss on the potato crop is estimated to about € 5 billion per year (Haverkort *et al.*, 2008). This pathogen is presently expanding its host range including other *Solanum* species like *S. nigrum* (night shade) and petunia (Flier *et al.*, 2003; Becktell *et al.*, 2006; Nelson, 2008).

1.2.1 *P. infestans* is not a fungus

P. infestans belongs to the oomycetes, a diverse group of eukaryotic organism, which include pathogens of plants, insects, crustaceans, fish, vertebrate animals, and microbes (Kamoun, 2003; Phillips *et al.*, 2008). Because of the filamentous nature previously oomycetes were considered as fungus-like organisms but in updated molecular phylogeny they are classified as stramenopiles (Burki *et al.*, 2007; Burki *et al.*, 2008; Beakes *et al.*, 2012). Beside oomycetes, photosynthetic brown algae and diatoms belong to the stramenopile organisms and oomycetes are thought to have evolved from phototrophic ancestors. Oomycetes differ from fungi in a number of biological criteria. Oomycete hyphae are always nonseptate (Latijnhouwers *et al.*, 2003). They have a diploid vegetative state whereas fungi generally are haploid. Oomycete mitochondria possess tubular cristeae opposed to flattened cristae in fungi (Taylor, 1978). Further, fungal cell walls are commonly composed of

chitin but oomycete cell walls are composed of cellulose and β-1,3 glucans (Beakes *et al.*, 2012). In a recent study, 3 different cell wall types (type I, type II and type III) are suggested for the two major orders, the Peronosporales and the Saprolegniales in the oomycete class (Melida *et al.*, 2013). *P. infestans* belongs to the Peronosporales and possess the type I cell wall, which is devoid of *N*-acetylglucoseamine but contains glucoronic acid and mannose.

The genus *Phytophthora* comprises over 100 species that are able to infect different plant species (Kroon *et al.*, 2012). Some *Phytophthora* species have a wide host range such as *P. capsici* (Lamour *et al.*, 2012), while others have a narrow host range such as *P. sojae* (Kaufmann and Gerdemann, 1958). *P. infestans* has both sexual and asexual stages in its life cycle (Fig. 2). Sexual mating in *P. infestans* enables genetic recombination leading to rapid adaptation to a changing environment.

1.2.2 Infection processes of *P. infestans*

P. infestans is a hemibiotroph using both biotrophic and necrotrophic phases to complete its infection cycle. The plant infection process of *P. infestans* is well known (Judelson and Blanco, 2005; Fry, 2008). In brief, the infection is initiated when sporangia germinate by forming invasive hyphae or by releasing motile zoospores (Tyler, 2002). Zoospores swim to reach the plant surface and upon shedding their flagella they attach themselves via secretion of adhesion molecules. Germinating zoospores form germ tubes, which swells at the tip to differentiate into an appressorium (Latijnhouwers *et al.*, 2003). Normally, oomycete appressoria are involved in penetration of the outermost, epidermal cell layers. After the plant cuticle and cell wall have been breached, an intracellular, biotrophic infection vesicle is produced in the epidermal cell (O'connell and Panstruga, 2006; Whisson *et al.*, 2007). Intercellular hyphae grow into the mesophyll cell layers, producing intracellular, biotrophic haustoria as new host cells are encountered. A haustorium is a nutrient uptake structure but not formed in all oomycetes; as for example, it is not found in

Aphanomyces euteiches. After 36–48 hours post inoculation (hpi), the mode of interaction between *P. infestans* and the initially infected plant cells becomes necrotrophic (Avrova *et al.*, 2008). From 72 to 96 hpi, initially infected areas of the leaf are fully colonized and necrotic and sporulating hyphae emerge from stomata to release sporangia. The sporangia then release zoospore and infect new host cells, and the process continues with new rounds of infected cells.

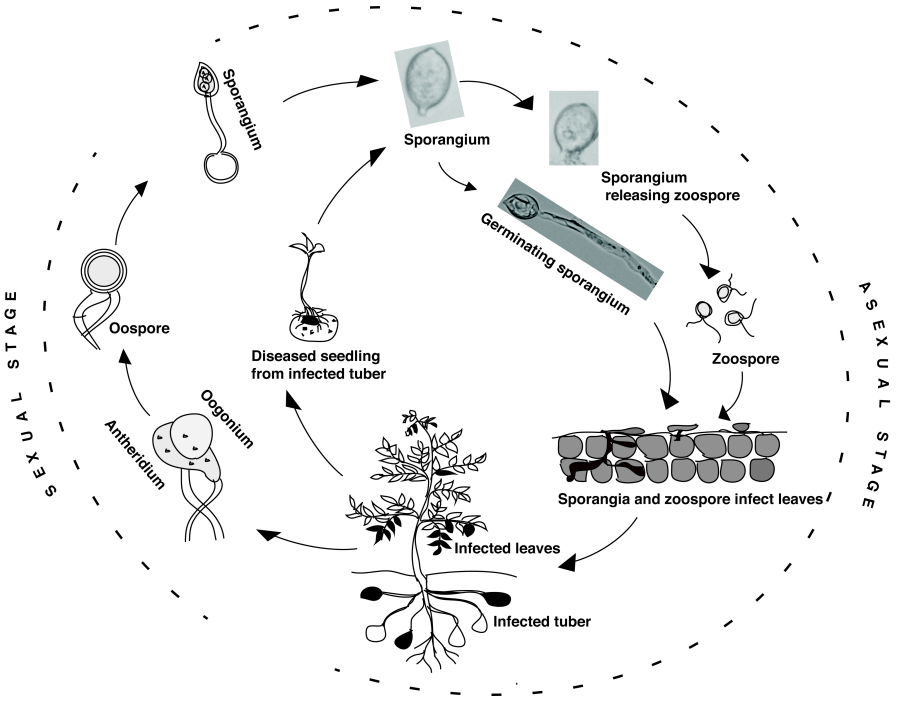

Figure 2. Different life cycle stages of *P. infestans*. Sporangia are carried through the wind. In cool and wet conditions they produce biflagellated zoospores, which can move a short distance. In contact with the host tissue they germinate to form a germ tube which penetrate the host tissue and form appressoria structures. Intercellular hyphae grow into the mesophyll cell layers, producing intracellular haustoria. When new host cells are encountered, more sporangiophores are formed and the infection cycle continues. Sexual reproduction is also present in *P. infestans.* When the A1 and A2 mating types are present in close proximity, fusion of oogonium and antheridium takes place, a process known as karyogamy. This leads to the formation of oospores, which will germinate to form sporangia and continue the infection cycle. Oospores are capable to survive in harsh conditions. For more details, see the reviews by Fry, (2008) and Fawke *et al.* (2015).

1.2.3 Effectors and evolution of *P. infestans* to overcome resistance

The genomes of several *Phytophthora* species are known (Table 2). The *P. infestans* genome is much larger than those of related species, which is attributed to the proliferation of repetitive DNA and transposons accounting for

Table 2. *Genome sequence of several Phytophthora species.*

Species	Genome size, Mb	Gene models	References
P. infestans	240	18,100	(Haas *et al.*, 2009)
P. capsici	65	12,011	(Lamour *et al.*, 2012)
P. ramorum	65	15,743	(Tyler *et al.*, 2006)
P. sojae	95	19,000	(Tyler *et al.*, 2006)
Hyaloperonospora arabidopsidis	99	14,543	(Baxter *et al.*, 2010)
Albugo candida	45	15824	(Links *et al.*, 2011)

approximately 74% of the genome. The genome of *P. infestans* is organized in an unusual pattern comprising a gene rich region that is densely packed and a gene sparse region, containing transposable elements (TE) and repetitive sequences (Tyler *et al.*, 2006; Haas *et al.*, 2009). The genes encoding effectors are mostly located in these gene sparse TE-dense regions. The mobile elements in the gene sparse region are thought to be associated with effector expansion and genome re-organizations and in that way contribute to the evolutionary plasticity of *P. infestans* in terms of pathogenicity and host specificity (Haas *et al.*, 2009; Grandaubert *et al.*, 2014).

Effectors are secreted pathogen proteins and other molecules that modulate plant defense system and enable parasitic colonization of plant tissues. *P. infestans* possesses large numbers (>700) of effector proteins including both apoplastic and cytoplasmic types (Kamoun, 2006). Most of the oomycete effectors possess an N-terminal signal peptide for secretion from the microbe. An exception is the *P. sojae* effector PsIsc1, which lacks the predicted secretory peptide (Liu *et al.*, 2014). After secretion, the apoplastic effectors function in the extracellular spaces and interfere with apoplastic plant defense

(Schornack *et al.*, 2009). In *P. infestans* they are mainly small cysteine-rich proteins such as, EPI1, a serine protease inhibitor that inhibits pathogenesis-related P69B subtilisin-like serine protease of tomato in intercellular fluids (Tian *et al.*, 2004). Haustoria are suggested to be the site from which cytoplasmic effectors translocate to host cells (Petre and Kamoun, 2014). The cytoplasmic RXLR and CRN (crinkler) effectors need to cross the plant cell wall and the plasma membrane or the extra-haustorial matrix and membrane to enter the plant cell (Schornack *et al.*, 2009). The RXLR (arginine-any amino acid-leucine-arginine) motif following the N-terminal signal peptide of the RXLR effectors is required for the translocation process (Rehmany *et al.*, 2005; Whisson *et al.*, 2007). The RXLR motif of the effector binds to surface-exposed phosphatidylinositol-3-phosphate (PI3P) to mediate effector translocation through endocytosis (Kale *et al.*, 2010). It has also been shown that RXLR- PI3P binding takes place inside the pathogen for stabilization and secretion of the effector (Yaeno *et al.*, 2011). There are also contradictory findings to this N-terminal RXLR-PI3P binding, suggesting PI3P binding to the C-terminal domain of the effector e.g. AVR3a (Wawra *et al.*, 2012). With all these contradictory findings the concept of a conserved host-targeting domain of the effector is under question mark.

After translocation into the plant cell the cytoplasmic effectors interact with host intracellular proteins and modulate its immune response (de Jonge *et al.* 2011). AVR3a, a RXLR effector in *P. infestans* stabilizes potato E3 ubiquitin ligase, CMPG1 and inhibits INF1 triggered ROS accumulation and cell death (Bos *et al.*, 2010). Another RXLR effector in *P. infestans*, PexRD2 interacts with potato kinase domain MAPKKKε, a positive regulator of cell death associated with host immunity (King *et al.*, 2014).

Effectors are suggested to be an adaptation to facilitate biotrophy due to the observation that RXLR effectors are induced during pre-infection and the biotrophic phases of the infection (Schornack *et al.*, 2010). In contrast the CRN effectors, inducing crinkling and necrosis, are suggested to be an adaptation to

necrotrophy such as seen in *Pythium* spp. (Levesque *et al.*, 2010). Although many oomycete species secrets both RXLR and CRN effectors making it difficult to correlate effector class and lifestyle.

1.3 The plant immune system

Plants rely on innate immune responses to recognize a wide range of pathogens The first line of defense occurs on the plant cell surface and comprises recognition of conserved pathogen or microbe associated molecular patterns (PAMP/MAMPs) (Chisholm *et al.*, 2006). These PAMPs are perceived by plant pattern recognition receptors (PRRs), which trigger different intracellular responses including rapid ion fluxes across the plasma membrane, MAP kinase activation, production of reactive oxygen species, rapid changes in gene expression and cell wall reinforcement leading to PAMP triggered immunity or PTI (Zipfel, 2008; Zipfel, 2014). For example Pep-13, a surface exposed stretch of a trans-glutaminase protein is a PAMP in *P. sojae,* which is recognized by the plant (Brunner *et al.*, 2002). Other PAMPs in *Phytophthora* species include INF1, a sterol binding protein secreted from *P. infestans;* OPEL, a secreted protein and CBEL (cellulose binding elicitor lectin) from *P. parasitica*; β-glucans, cell wall fractions from fungi and oomycete and many others (Zipfel, 2014; Fawke *et al.*, 2015). Successful pathogens evolved effectors to interfere with the PTI response resulting in effector-triggered susceptibility (ETS). In turn, plants counteract and evolve resistance proteins to recognize specific effectors directly or indirectly, causing effector-triggered immunity (ETI). ETI often causes a hypersensitive cell death response (HR). In this “arms-race”, pathogens evolve new effectors to avoid or suppress ETI. The mostly referred model to explain these plant-pathogen interaction events is the four-phased ‘zigzag’ model (Jones and Dangl, 2006). This model adapts to the previously formulated gene-for-gene hypothesis developed by Flor in 1956.

Different updated models of plant immune responses along with their limitations are discussed by Cook *et al*. (2015).

1.3.1 Resistance proteins in plants against pathogen

In the plant immune system, pathogen recognition relies on two groups of receptors (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Glowacki *et al.*, 2011). The first group comprises PRRs, which are generally receptor-like proteins or receptor-like kinases (RLPs or RLKs), attached to the cell surface. The receptor-like protein, ELR (elicitin response) from wild potato *S. microdontum* is a recently identified host protein, able to recognize conserved molecular pattern of *Phytophthora* species (Du *et al*. 2015). Association of ELR with the co-receptor BAK1/SERK3 mediates recognition of four diverse elicitins (INF1, INF2A, INF5 and INF6) from *P. infestans* and induces cell death in the plant. The second group of receptors consists of intracellular receptors known as resistance proteins or R proteins. Most of these R proteins are members of the nucleotide binding site-leucine rich repeat (NB-LRR or NL) protein family. NB-LRR proteins are highly abundant in plant genomes (Hulbert *et al.*, 2001; McHale *et al.*, 2006; Marone *et al.*, 2013). The NB domain and LRRs have a very ancient origin since several genes in the eubacteria are identified encoding either a NB domain or LRRs (Yue *et al.*, 2012; Jacob *et al.*, 2013). The fusion between NB and LRR is not observed until evolution of mosses. The NLs are further subdivided into two subclasses, based on the N-terminal structural features. The first subclass contains an N-terminus referred to as the *Drosophila* Toll and mammalian interleukin-1 receptors or TIR (TNL), while the second subclass contains a coiled-coil (CC) domain (CNL). It is predicted that the combination of the TIR domain with the NL has evolved earlier than its CNL counterpart (Yue *et al.,* 2012).

These two *R*-gene subfamilies differ in their structures and in signaling pathways. In the *Arabidopsis thaliana* genome 150 NB-LRR encoding genes are present, in *Oryza sativa* (rice) more than 400 are found and in diploid *S.*

tuberosum 738. In *A. thaliana* 83 represent TNLs and 51 are CNLs and in potato 448 NB-LRR proteins belong to the CNL group and 288 to TNL group whereas in grass species TNLs are absent (McHale *et al.*, 2006; Bakker *et al.*, 2011). The NB domain is required for ATP or GTP binding (Tameling *et al.*, 2006). Conformation changes of the NB domain caused by reversible nucleotide binding, leads to the activation/deactivation of the whole receptor (Tameling *et al.*, 2006). On the other hand, the LRR domain seems to mediate specificity in pathogen recognition through specific protein-protein interaction (Bella *et al.*, 2008), while the N-terminal TIR or CC motif is likely to play a role in downstream signaling (Ellis *et al.*, 2000; Yi and Richards, 2007). Sequence polymorphism affecting pathogen specificity is mostly observed in the LRR-coding region, (Ellis *et al.*, 2000)

There are different models regarding evolution of plant resistance genes (Bergelson *et al.*, 2001). In one model the host disease resistance loci are considered to be monomorphic and under selection pressure, enabling stronger interaction with the particular effectors (Stahl *et al.*, 1999). This model necessitates a series of selective sweeps enabling *R* gene alleles to recognize pathogenicity determinants. The evolution of amino acids in the functionally important region of resistance proteins happens quite fast (Bergelson *et al.*, 2001). According to the population genetics theory of selective sweeps this should lead to reduction in the age and number of alleles at a given *R*-gene locus. But the longevity and high allelic diversity of some *R*-loci in plant populations suggests micro-evolutionary mechanism promoting the maintenance of stable polymorphism (Bergelson *et al.*, 2001).

1.3.2 Exploitation of *R* genes to develop resistant plants

Attempts to achieve durable resistance in important crop plants have been ongoing for a long time. Both classical breeding and transgenic approaches have been used. Resistance breeding program in potato against *P. infestans* started during the first half of the 20th century (Biffen 1905), involving

utilization of dominant *R* genes from the wild species like *S. demissum* (Vleeshouwers *et al.*, 2011). The identified *R* genes in *S. demissum* today are *R3 (R3a, R3b), R5, R6, R7, R8, R9, R10, R11* on chromosome 11, *R2* on chromosome 4 and *R1* on chromosome 5 (Elkharbotly *et al.*, 1994, 1996; Leonardsschippers *et al.*, 1994; Bradshaw *et al.*, 1998; Li *et al.,* 1998*;* Huang *et al.,* 2004). Introgression of these *R* genes into new commercial varieties was initially successful but the resistance was rapidly overcome by the genetic evolution of the pathogen (McDonald and Linde, 2002; Fry, 2008). The breeding materials also suffered from linkage drag and introduction of many unwanted traits (Jacobsen and Schouten, 2007; Park *et al.*, 2008). For example, Toluca and Bionica are two potato cultivars generated by sexual introgression of *Rpi-blb2* from *S. bulbocastanum* for durable resistance against late blight (Haverkort *et al.*, 2009). A work based on more than 30 years of crossings and intense selection. Cloning of *R* genes and transformation to crops is the fastest and easiest way of introgression of desired traits into an advanced genomic background (Jones *et al.*, 2014). Crops generated this way are known as genetically modified (GM). Examples of different GM crops with disease resistance are summarized by Dangl (2013). Fortuna is a resistant potato cultivar developed by the BASF company using GM technologies. Fortuna showed excellent field resistance against *P. infestans* (Jones *et al.,* 2014). Compared to Toluca and Bionica, Fortuna was developed in a much shorter time and also carried two resistance genes (*Rpi-blb1 and Rpi-blb2*). However, considering high cost in marketing this cultivar in Europe, further investment to this project has been withdrawn. In parallel to GM approaches, various breeding programs have an emphasis on broad-spectrum resistance to *P. infestans*. Here the development of the Sarpo Mira cultivar harboring *R3a, R3b, R4,* and the *Rpi-Smira1* genes is a good example (Rietman *et al.*, 2012). This cultivar fulfills criteria for organic growers but suffers from a number of unwanted traits making it suitable for a larger market. Today the resistance in Sarpo Mira also starts to break down.

1.4 Non-coding small RNAs

RNA is a key player in molecular biology encompassing both informational and regulatory functions (Mattick and Makunin, 2006; Morris and Mattick, 2014). Since the middle of 20th century the major function of RNA was emphasized on the physical link between DNA and protein. But rather than performing just a messenger role, RNAs are performing much more important functions. Recent studies suggest that the major part of mammalian and other genomes of complex organisms is transcribed into small (sRNA) and long noncoding RNAs (Mattick and Makunin, 2006). sRNAs are playing important regulatory role in disease and developmental biology through RNA silencing (Bonnet *et al.*, 2006; Vazquez *et al.*, 2010; Pauli *et al.*, 2011).

1.4.1 Biogenesis, classification and mechanism of sRNAs in plants

sRNA mediated RNA silencing processes and the different sRNA classes involved have been extensively studied in model species such as *A. thaliana, Drosophila melanogaster, Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Ghildiyal and Zamore, 2009; Malone and Hannon, 2009; Ruiz-Ferrer and Voinnet, 2009; Axtell, 2013). The comprehensive classification of sRNAs in *A. thaliana* is presented in Fig. 3. Among different types, microRNAs (miRNAs) and the small interfering RNAs (siRNAs) are the major class of sRNAs. siRNAs can be generated from both endogenous and exogenous source of double-stranded RNA precursors. miRNAs are generated from endogenous single stranded precisely processed hairpin RNA. siRNAs are further subdivided into heterochromatic siRNA, secondary siRNA and natural antisense transcript siRNA (NAT-siRNA). Secondary siRNAs can be further classified as phased and *trans*-acting and the NAT-siRNAs can also be *cis*-acting or *trans*-acting. sRNAs are coordinating the expression, stability, protection and inheritance of eukaryotic genomes both at transcriptional or post-transcriptional levels (Castel and Martienssen, 2013; Holoch and Moazed, 2015). One important class of sRNA, Piwi-associated RNAs (piRNAs) are

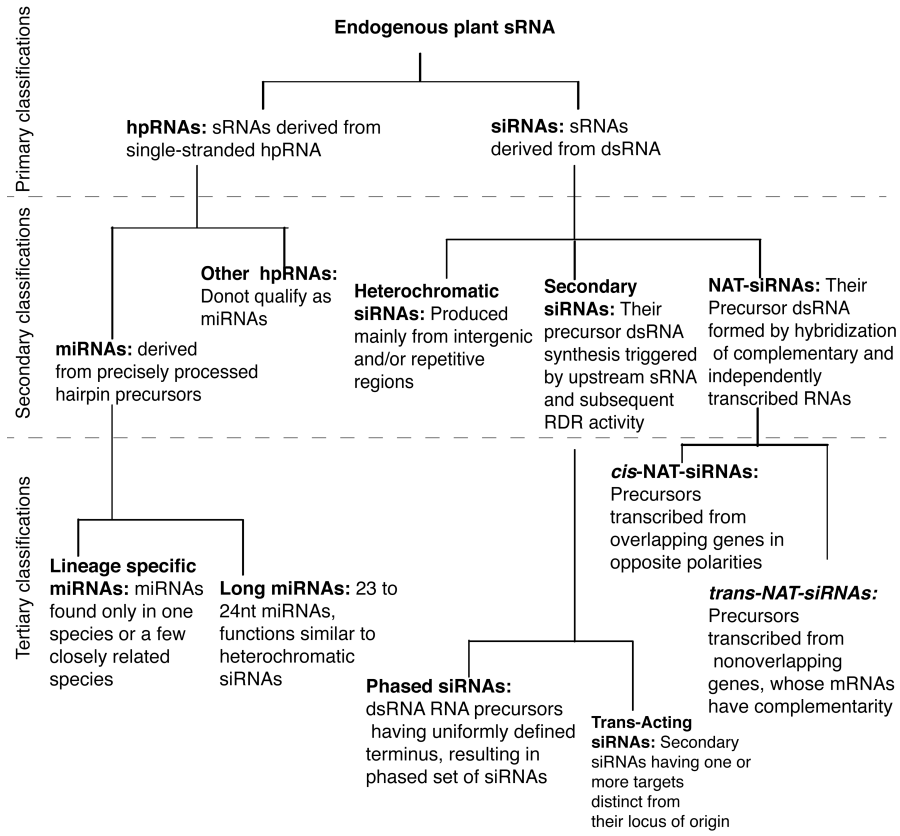

Figure 3. Hierarchical classification of endogenous small RNAs in *A. thaliana*. Solid black lines are indicating hierarchical relationships. Abbreviations: sRNAs, small RNAs; dsRNA, double-stranded RNA; hpRNA, hairpin RNA; miRNA, microRNA; NAT-siRNA, natural antisense transcript small interfering RNA; siRNA, small interfering RNA. Adapted from Axtell (2013).

found only in animal and are involved in the regulation of transposable elements in the germline (Malone and Hannon, 2009).

The biogenesis and mechanism of siRNA and miRNA mediated gene silencing in *A. thaliana* is presented in Fig. 4. sRNAs are often ranging in size from 19–40 nt. The dsRNA from different sources is processed by one of four ribonuclease III type protein called dicer or dicer like protein (Dcl) into short duplexes known as siRNA (short interfering RNA) (Deleris *et al.*, 2006; Henderson *et al.*, 2006; Liu *et al.*, 2009). miRNAs are transcribed from

miRNA encoding genes into pri-miRNA (Chen, 2005). Pri-miRNA is processed into pre-miRNA and then to mature miRNA. In *A. thaliana* these two steps are carried out by Dcl1 proteins in the nucleus (Denli *et al.*, 2004) but in animals Drosha carries out the former step. The siRNA and miRNA duplexes are protected from degradation by the methyltransferase HUA ENHANCER 1 (HEN1) through *2´-O*-methylation (Boutet *et al.*, 2003; Yu *et al.*, 2005). Both the siRNA and miRNA duplex is unwound and the antisense strand incorporated to one of ten Argonaute (AGO) proteins to form RNA induced silencing complex (RISC) (Vaucheret, 2008). Normally the mature miRNAs are loaded into AGO1. The RISC then targets mRNA complementary to siRNA or miRNA and degrades it leading to post transcriptional gene silencing (PTGS) by endonucleolytic cleavage or by translational repression (Czech and Hannon, 2011). RNA dependent RNA polymerases (RdRP) are involved in amplification of silencing by the production of dicer substrate double-stranded RNA. siRNA can also induce DNA methylation and chromatin modification of the target loci leading to transcriptional gene silencing (TGS) (Law and Jacobsen, 2010). RNA polymerase IV and V are associated with RNA directed DNA methylation. RNA polymerase IV generates single stranded RNA (ssRNA) transcripts, which correspond to transposons and repeat elements (Law and Jacobsen, 2010). A putative chromatin-remodelling factor CLASSY 1 (CLSY1, also known as CHR38) is suggested to recruit Pol IV to chromatin or to aid in ssRNA transcript processing. From this ssRNA, RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) generates long perfectly complementary dsRNA, which is processed by Dcl3 into 24 nt siRNA. These siRNAs are primarily loaded into AGO4 and secondarily into AGO6 or AGO9 and initiate PolV mediated de novo DNA methylation ((RdDM) through DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) along with some other dedicated proteins (Law and Jacobsen, 2010; Matzke *et al.*, 2015).

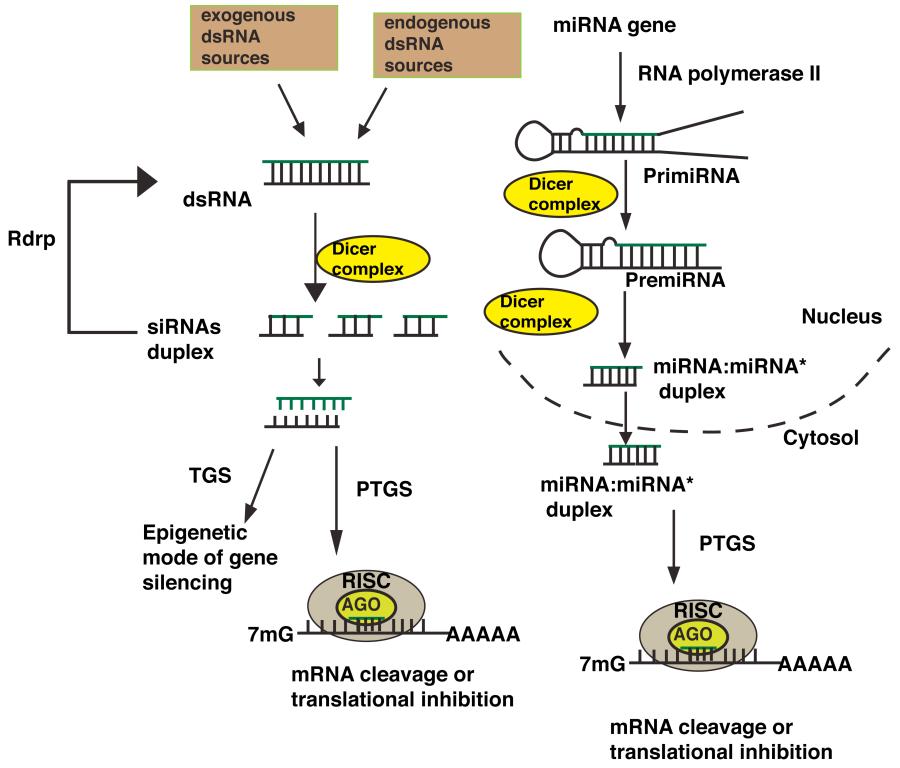

Figure 4. Schematic presentation of biogenesis and mechanism of action of siRNA and miRNA in *A. thaliana*. In the siRNA pathway double stranded RNAs (dsRNAs) from different sources are processed by the Dicer complex into siRNA duplexes. This siRNA duplex unwinds and one of the strands gets incorporated in to the AGO and form RISC (the RNA induced silencing complex). This RISC can then mediate TGS (transcriptional gene silencing) or PTGS (post transcriptional gene silencing). TGS involves histone modification or DNA methylation. PTGS involves RNA degradation or translational repression. miRNA genes are transcribed by RNA polymerase II forming pri-miRNA, which is processed by the Dicer complex to pre-miRNA and further processed by Dicer complex to the miRNA:miRNA* duplex. This whole processing of mature miRNA duplex from pri-miRNA is carried out in the nucleus, in contrast to animal. This duplex is transported in to the cytoplasm where it unwinds and one of the strands gets incorporated into AGO, forming the RISC. This complex then recognizes the target mRNA and causes mRNA cleavage or translational inhibition. Adapted from (Sarkies and Miska, 2014).

There are additional proteins associated with RNA silencing. RNA helicases bind or remodel RNA or RNA–protein complexes in an ATP-dependent fashion. Whereas cytosine methyltransferase, histone methyltransferase, histone deacetylase, and chromodomain proteins are involved in sRNA

mediated transcriptional gene silencing through cytosine methylation or histone modifications.

1.4.2 Role of endogenous small RNAs in plant immunity

Small RNAs like siRNAs and miRNAs are playing important role in plant immune responses against a wide range of pathogens and insects (Pelaez and Sanchez, 2013). Plant defense responses are under tight regulation and small silencing RNAs act as modulator of these defense responses (Voinnet, 2008; Katiyar-Agarwal and Jin, 2010). Under normal conditions, plant defense responses are suppressed but need to be turned on promptly during pathogen attack. Endogenous sRNAs are performing the 'on' and 'off' switch through reprogramming and fine-tuning the immunity gene expression by silencing the negative regulators or inducing positive regulators (Navarro *et al.*, 2006). For example in *A. thaliana*, when sensing flagellin (a PAMP from *Pseudomonas syringae)* enhanced transcription of miR393 is induced, which then targets mRNAs encoding F-box auxin receptor transport inhibitor response1 (TIR1) and related proteins. Thus activation of miR393 leads to PAMP triggered immunity against *P. syringae* through negative regulation of the auxin signaling pathway (Navarro *et al.*, 2006).

A handful of examples are known on sRNAs in plant immunity including *Solanaceae* plants. Six miRNAs are reported in tobacco, tomato and potato, which are involved in targeted cleavage of functional *R* gene transcripts. In tomato miR482 controls the expression of CC-NB-LRR disease resistance proteins through the activation of a silencing cascade (Shivaprasad *et al.*, 2012). Further, RNA silencing can activate defense responses in *A. thaliana* against *Hyaloperonospora parasitica* (Yi and Richards, 2007). In this context miR393 acts as positive regulator of soybean defense against *P. sojae* due to impacts on the isoflavonoid biosynthetic pathway (Wong *et al.*, 2014) and knock-down of miR393 in soybean leads to hyper-susceptibility to *P. sojae.* To counter-act the sRNA regulated immune system in plants, pathogens have

evolved silencing suppressors (Xiong *et al*. 2014). In *P. sojae* two effectors are identified which are designated as *Phytophthora* suppressor of RNA silencing (PSRs). *P. sojae* PSR2 (PsPSR2) is shown to suppress RNA silencing in soybean and promote infection. (Xiong *et al.*, 2014). Plants have also evolved defense against the pathogen-mediated silencing suppressor (Pumplin and Voinnet, 2013) and guard the integrity of key silencing components to trigger ETI (effector triggered immunity). For example, viral suppressor of RNA silencing 2b (VSR 2b) from *tomato aspermy* cucumovirus elicits an HR upon expression in tobacco by a modified TMV strain (Li *et al.*, 1999).

Generation of small RNA in plants, induced by pathogen infection, can alter DNA methylation and chromatin modification leading to activation of certain genes (Katiyar-Agarwal and Jin, 2010). Truncated DNA transposons such as MITES (miniature inverted repeat transposable elements) are common (Jiang *et al.*, 2004). They are present in several plant genomes in high copy numbers and are involved in evolution of the plant immune system through generation of sRNAs (24 nt) (Kuang *et al.*, 2009). MITES are identified within the *R* gene regions of tomato, potato and tobacco. In tobacco, they are shown to maintain the complexity of TMV *R* gene *N* through regulation of its expression at transcriptional and/or posttranscriptional level. Transient modification of TGS (transcriptional gene silencing) mechanism induced by TMV infection may lead to changes in *N* transcription. Also TMV infection may temporarily induce inhibition of PTGS (post transcriptional gene silencing) for *N* expression. This may lead to gene diversity through introducing insertions or deletions or by inducing premature translational inhibition.

1.4.3 Transport of RNA silencing signal

Plant has specialized mechanism to maintain cell-to-cell communication and also for transport of signals between a plant and pathogens (Kehr and Buhtz, 2008). sRNA-mediated gene silencing is an important defense mechanism in plants and the silencing signal must be transported within the plant and also to

the pathogen and vice versa. Previous work involving grafting experiments on transgenic plants has shown the transmission of a silencing signal from the silenced rootstock to the non-silenced scions (Palauqui *et al.*, 1998). For both long-range and short-range transport the mobile silencing signals are suggested to be siRNA or siRNA precursor (Hamilton *et al.*, 2002). The local movement of silencing signal occurs symplastically involving plasmodesmata and the long distance transport occurs through the phloem (Melnyk *et al.*, 2011). Both 21 and 24 nt siRNA duplexes are suggested to move through plasmodesmata and phloem (Dunoyer, 2010; Molnar *et al.*, 2010; Melnyk *et al.*, 2011). So far only 21 nt siRNAs seems competent to move (Dunoyer, 2010). Other factors than size could also be the determinant for sRNA local or systemic movement; such as, the origin of the sRNAs e.g. genetic locus or the size of the sRNA precursor (Melnyk *et al.*, 2011). In *A. thaliana* miR165 and miR166 can move over short distances (Zhou *et al.*, 2007). This is further evident by accumulation of mature miRNA in the outer layer of the root while, the activity of the promoter driving their expression is highest in the inner area of the root (Carlsbecker *et al.*, 2010). Long-range movement of miR399 from shoot to root has also been demonstrated (Lin *et al.*, 2008; Pant *et al.*, 2008). Like siRNAs, miRNAs are suggested to be transported as miRNA duplex (Buhtz *et al.*, 2008). In case of miRNAs and their cell-to-cell movement and the long distance transport in the plant vascular system, a 27 kD small RNA-binding protein is required (Kehr and Buhtz, 2008).

The complex regulatory mechanism of RNA trafficking and transport of proteins between plant and the pathogen is mostly unknown. It remains to be shown how RNA, DNA, and proteins are delivered from the plant, across the plasma membrane and plant cell wall into pathogen or vice versa. Is the process active, passive and what components are involved? In other words, we know what is present on either side but do not know how the components are translocated.

1.4.4 RNA silencing in *P. infestans*

P. infestans possesses an active RNA silencing pathway (Vetukuri *et al.*, 2011a). It has genes encoding the major components of the RNA interference (RNAi) pathway, Dicer-like (PiDcl1), Argonaute (PiAgo1–5) and RNA-directed RNA polymerase (PiRdr1), together with genes potentially involved in gene silencing, such as histone deacetylases, histone methyltransferases, DEAD helicases, chromodomain proteins and a class 1 RNaseIII. A second dicer-like gene *PiDcl2* was later found in the genome trace archive (Fahlgren *et al.,* 2013). *PiDcl2* is similar to *P. sojae Dcl2,* harboring only two RNaseIII domains (Matari and Blair, 2014). While PiDcl1 has two RNase III domains and a double stranded RNA binding domain this protein has also a nuclear localization signal (Vetukuri *et al.*, 2011a). According to domain organization, PiDcl1 relates to *Schizosaccharomyces pombe* Dcr1 and *Neurospora crassa* Dcl1, and based on sequence similarity PiDcl1 is more similar to *A. thaliana* DCL4 protein. Genes encoding components of gene silencing involved in DNA methylation, such as cytosine methyltransferases and RNA polymerase IV, are not found in the *P. infestans* genome. Genes encoding proteins involved in miRNA biogenesis, such as Drosha (Lee *et al*., 2003) are lacking together with gene homologues of ERI1 involved in 5.8S ribosomal RNA processing (Ansel *et al.,* 2008; Gabel and Ruvkun, 2008) and HEN1. Among five Argonaute proteins in *P. infestans* PiAgo1 and 2 are identical, have nuclear localization signal and probably involved in transcriptional gene silencing (Vetukuri *et al*. 2011a).

Gene silencing in *P. infestans* could be post-transcriptional occurring downstream of transcription and transcriptional taking place upstream of transcription resulting in transcriptional arrest (Cogoni and Macino, 2000; Verdel and Moazed, 2005; Malecova and Morris, 2010). The silencing pathway in *P. infestans* acts to control the huge amount of transposons in its genome. The repetitive DNA of *P. infestans* comprises a wide repertoire of transposons: short interspersed elements (*SINEs*), *non-long-terminal repeat*

(non-LTR), long interspersed elements (LINEs), Copia and Gypsy LTR retrotransposons, Cryptons, Helitrons, DIRS-like, mini-transposable elements (MITEs), hATs, PiggyBACs, Mutators, Mariners, and a broad diversity of novel *LTR* and *DNA transposons* (Haas *et al*., 2009). In *P. infestans* TEs are controlled by heterochromatin formation (van West *et al*. 2008). Also sRNAs derived from all families of transposons, emphasizing the importance of RNA silencing in maintaining TEs in an inactive form (Vetukuri *et al.,* 2013). Effector genes are usually located within 600-2000bp from TEs (Whisson *et al.*, 2012). It is speculated that silencing of TEs probably regulates the expression of the effector genes in close proximity.

2 Aims of the study

In this thesis, I aimed to study different sRNA populations in different isolates of *P. infestans* with varying pathogenicity and initiate studies on their potential cross-talk with host plants. For the later purpose, I attempted to study both sRNAs and transcripts in potato during interaction with *P. infestans*. I also aimed at implementing the knowledge of trans-kingdom RNAi to impact the immunity in potato against *P. infestans*. The long-term goal of this thesis work is to enhance our understanding of the interaction events taking place between *P. infestans* and potato, and hopefully to use such knowledge in new resistance breeding strategies.

Specifically the aims of my different projects were to:

- Characterize different classes of sRNAs and their biogenesis in *P. infestans*.
- Investigate the involvement of *P. infestans* sRNAs in pathogenicity.
- Profile potato and *P. infestans* transcripts during their interaction.
- Profile potato miRNA and investigate their role in host gene and regulation during potato-*P. infestans* interaction.
- Investigate the possibility of exploiting RNA silencing to target selected genes in *P. infestans* via the host plant, and thereby impacting the defense response against *P. infestans*.

3 Results and Discussions

3.1 Small RNA repertoire of *P. infestans* and their association with pathogenicity (Paper I and II)

The gene silencing system has been studied in *P. infestans* and shown to possess both transcriptional and post-transcriptional gene silencing (TGS and PTGS). Expression of sense and antisense gene constructs followed by nuclear run-on assays, suggest the activation of TGS processes (van West *et al.*, 1999; Judelson and Tani, 2007) and the treatment of protoplasts with dsRNA, showed transient RNA silencing in *P. infestans*, most probably via the PTGS machinery (Whisson *et al.*, 2007). Gene silencing has been utilized as tool to study roles of different genes in life cycle stages and during plant infection (van West *et al.*, 1999; Ah Fong and Judleson, 2003; Avrova *et al.*, 2008; Vetukuri *et al.*, 2011b).

Transient silencing of *PiDcl1* (*P. infestans* dicer like protein 1), *PiAgo1/2* (*P. infestans* Argonaut 1/2) showed evidence for Dicer-dependent and Argonaute-dependent silencing pathways in *P. infestans.* Dicer-dependent pathway is responsible for the generation of 21 nt sRNAs and Argonaute dependent pathway is associated with the generation of longer sRNAs (Vetukuri *et al.*, 2012). 32 nt sRNAs from the *Avrblb1* effector encoding gene were shown to be negatively affected by *PiAgo4* and *PiAgo5* depletion. However, upon identification of PiDcl2 it was suggested to be responsible for

the generation of 25/26 nt sRNAs (Fahlgren *et al.*, 2013). The Argonaute proteins in *P. infestans* are also suggested to be grouped into two or more groups probably responsible to stabilize 21 or 25/26 nt sRNAs. Moreover Argonautes showed base preference at the 5´nt of sRNAs (Åsman *et al.,* 2015). PiAgo1 is bound to 21 nt sRNA with 5´C and Ago4 to 25 nt sRNA with 5´U preferences.

Deep sequencing of RNA from four life cycle stages; mycelium, sporangium, germinating sporangium and germinating cysts of two different isolates of *P. infestans* with contrasting infection abilities (R0, weakly pathogenic and 3928A, highly pathogenic) revealed diverse classes of sRNAs in *P. infestans* mapping to effector encoding genes, transposons and tRNAs (Vetukuri *et al.,* 2012; Åsman *et al.,* 2014). The most abundant size classes of sRNAs are 21 nt and 25/26 nt. This agrees with the findings of bimodal size profile of sRNAs in *P. infestans, P. sojae and P. ramorum* with peaks on 21 and 25 nt RNAs (Fahlgren *et al.* 2013). Other sRNA size classes in *P. infestans* involve 31, 32, 35 and also 40 nt. The existence of variable size class of sRNAs in *P. infestans* is in contrast to abundance of a single size class of sRNAs in *D. melanogaster*, *Dictyostelium discoideum*, *Toxoplasma gondii* and *Cryptococcus neoformans* (Li and Godzik, 2006; Hinas *et al.*, 2007; Ghildiyal *et al.*, 2008; van West *et al.*, 2008; Wang *et al.*, 2010). All size class (19-33 nt) of sRNAs from our data set mapped to RxLR effector genes and the 21 nt sRNAs predominately mapped to CRN effector genes. A majority of the sRNA population of *P. infestans* maps to TEs, and specially to the long terminal repeat (LTR) retrotransposons. The majority of sRNAs that maps to TEs are of 21 and 25/26 nt length but Northern hybridization has detected 32 and 35 nt sRNAs from *Crypton6* and *Gypsy-Pi1*.

We found differences in relative abundance of sRNAs mapping to some effector genes when comparing the R0 and 3928A isolates of *P. infestans.* There was higher proportion of 31-33 nt sRNA derived from TE and RxLR encoding genes in the R0 isolate. For example, more abundant sRNAs

homologous to *PiAvrblb2, PiAvr3a* and *PITG_14783* genes in the R0 isolate compared to 3928A. In contrast, lower transcript levels of corresponding effector genes were detected in both isolates by performing qRT-PCR in infected leaf samples. The transcript level of *PiAvr3a* was approximately 277-fold lower in the R0 isolate compared to 3928A. Both *PiAvrblb2* and *PiAvr3a* are known to be essential pathogenicity factors in *P. infestans* ((Bos *et al.*, 2010; Bozkurt *et al.*, 2011). The difference in relative abundance of mRNA and sRNA level for specific effector molecules between weakly pathogenic (R0) and pathogenic (3928A) isolates are allied to their respective infection phenotypes on the host plant. Similarly, in *P. sojae* more sRNAs were identified in a strain with a non-detectable *Avr3a* gene (Qutob *et al.*, 2013). This strain of *P. sojae* could escape detection by *Rps3a* of the host and are virulent.

For the effector *PiAvr3b,* lower transcript level along with higher abundance of sRNAs for this gene was detected in the 3928A isolate. This lower transcript level is probably in advantage to evade detection by the plant resistance gene *R3b.* A similar response is seen in *P. sojae*, where *PsAvr3b* evades detection by the resistance gene *Rps3b* through sequence variation and reduced transcript accumulation (Dong *et al.*, 2011). Transient silencing of *PsAvr3b* lead to compromised virulence on the susceptible soybean cultivar. These findings reflect the possible role of sRNA-mediated gene silencing as regulators of pathogenicity.

Six miRNA candidates ranging in size 21-24 nt, along with their targets were identified (Vetukuri *et al.*, 2012). They share features of both plant and metazoan miRNAs. Today, a single experimentally verified miRNA, miR8788, is reported from three species (*P. infestans, P. ramorum* and *P. sojae*) (Fahlgren *et al.*, 2013) and is 21 nt long in *P. infestans.* Presence of only single miRNA is in contrast to the numerous miRNAs found in plants (Nozawa *et al.*, 2012). The biogenesis mechanism of miRNA in *A. thaliana* is well studied (Rogers and Chen, 2013). The mature miRNAs are processed by the Dcl1

protein complex but an alternative mechanism for the biogenesis of miRNA has been shown for vertebrates (Cheloufi *et al.*, 2010). In this case the maturation of miRNA requires Ago instead of Dicer. The pre-miRNA is cleaved by Ago and generates intermediate 3´end that is further trimmed. The miRNA biogenesis and its mechanism of action in *P. infestans* are still to be unveiled.

An additional group of sRNAs, tRNA-derived RNA fragments (tRFs) ranging in size from 19-40 nt was also identified in *P. infestans* (Asman *et al.*, 2014). The majority of tRFs in *P. infestans* was 25-30 nt long and enriched for the sense strand. Northern hybridization could detect longer tRFs e.g. 40 nt 3´tRFs and 37 nt 5´tRFs which is not unlikely since our SOLiD sequencing had 30 nt as the upper read length limit. The precise sequence composition and abundant expression indicates individual tRFs as novel class of sRNAs rather than just random degradation intermediates during tRNA biogenesis and turnover (Megel *et al.*, 2015). In humans, plants and protists tRFs are generated by cleavage of the tRNA at the anticodon-loop to generate tRNA halves and in the D- and T-loops to generate 5´tRFs and 3´CCA tRFs (Lee and Collins, 2005; Hsieh *et al.*, 2009; Lee *et al.*, 2009; Sobala and Hutvagner, 2011). Another class of tRFs known as 3´ U tRFs is produced from the 3´end of pretRNA and ends in the RNA polymerase III termination poly-U tract (Lee *et al.*, 2009; Sobala and Hutvagner, 2011). Through Northern blot analysis, for tRNA Ile_cluster0 and Thr_cluster1 we detected the strongest signal for 5´half tRFs and for Arg_cluster0 the detected tRFs were the 5´and 3´tRNA halves and the internal fragment generated from cleavage of both anticodon- and T-loops.

In our sequenced data for two isolates (R0 and 3928A) and four developmental stages of *P. infestans* the 5´ tRFs are more abundant compared to the 3´ tRFs. On average, approximately 90% of tRFs were 5´tRFs and only around 4% were 3´ tRFs in both R0 and 3928A. The higher abundance of 5´ tRFs in *P. infestans* is comparable to the situation in *Trypanosoma cruzi,* where the nutritional stress-induced tRFs are mostly originated from 5´halves of

mature tRNAs (Garcia-Silva *et al.*, 2010). The tRFs in *P. infestans* mostly matches to the 5´part of tRNA Ile_cluster. Among total tRFs identified, 26% in R0 mycelia and 25% in 3928A germinating cyst mapped to this cluster. Abundance of tRFs from specific tRNAs suggest isolate or life cycle specific isoacceptor preferences. This is similar to *T. cruzi*, where the tRFs are reported to derive from $tRNA^{His}$, $tRNA^{Arg}$ and $tRNA^{Thr}$ (Franzen *et al.*, 2011).

Majority of tRFs of different size classes in our sequenced data showed enrichment for 5´G nucleotide indicating strong evolutionary conservation of the tRNA G_{+1} nucleotide, which is needed for RNaseP cleavage site recognition and successful pre-tRNA processing (Kirsebom, 2007). The 27 nt tRFs, 5´U nucleotide was more prevalent suggesting different process for the generation of these tRFs and also indicating that probably they are bound by distinct PiAgo complex.

The mechanism of tRNA biogenesis in *P. infestans* is not known. Involvement of Dcl and Ago in some tRFs generation has been shown for yeast, *Tetrahymena thermophile* and mammalian cells (Haussecker *et al.*, 2010). Hairpin RNA-mediated silencing of *PiAgo1* and *PiDcl1* genes was utilized to study their involvement in tRF biogenesis. No negative influence on production, and stability of different tRFs in *PiDcl1* knocked-down transformants compared to the wild type was observed by Northern hybridization and Illumina sequencing. PiAgo1 and PiAgo4 seemed to be associated with accumulation of specific tRFs, but how these Ago proteins are involved in cleavage of tRNAs is not well known. It could be speculated that PiAgo is performing endonuclease cleavage of tRNAs to generate tRFs or it may act by binding and stabilizing tRFs. In addition to Dcl and Ago, *P. infestans* possesses additional classes of endoribonucleases. Yeast Rny1p is a well-studied RNase T2 type eukaryotic tRNA cleavage nucleotide (Thompson and Parker, 2009). In the *P. infestans* genome five homologs of RNase T2 are predicted. Thus it could be speculated that tRNA cleavage enzyme in *P. infestans* could be of the RNase T2 type.

In order to study the tRFs population during the potato - *P. infestans* interaction, we sequenced potato leaves infected with 88069 and the PiDcl1 silenced transformant, sampled at 24, 48 and 72 hpi. Differential abundance of tRFs was observed in *P. infestans*. The relative proportion of accumulated 5´tRFs to 3´tRFs was elevated during host infection compared to in mycelia. Differential accumulation of some specific tRFs was observed during the infection process. For example there was a reduced level of 32 nt Ile0-5´tRFs during infection. On the other hand, higher level of tRFs from tRNA Pro_cluster1 during biotrophic (24 hpi) and transition from biotrophic to necrotrophic (48 hpi) phase of infection were seen. Proline is proposed to regulate osmotic pressure in *P. infestans*, which is important to penetrate the potato leaf cuticle (Grenville-Briggs *et al.*, 2005). During the preinfection stage (germinating cyst with appressoria) of *P. infestans,* 4 times elevation of free proline was observed compared to that of in mycelia. Differential level of Pro_cluster1 tRFs may reflect a mechanism where *P. infestans* regulates the proline levels in host infection.

3.2 Can a host-driven sRNA hairpins target pathogenicity genes in *P. infestans?* (Paper III)

Over the past years RNAi has been utilized as a tool for functional genomics (reviewed by Mohr and Perrimon, 2012). Sequence-specific knock-down of gene function enables assigning functions to specific genes involved in infection, cancer or different signalling pathways. RNAi has also been used to develop a strategy entitled host-induced gene silencing (HIGS) to improve resistance in plants against insect, parasitic nematodes, parasitic plants, bacteria, fungi and oomycetes. Different HIGS approaches along with their outcomes are reviewed by Koch and Kogel (2014). For example, it has been demonstrated that host-induced silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes in *Fusarium graminearum* species confers strong

resistance in both *A. thaliana* and *Hordeum vulgare* (barley) against this fungus (Koch *et al.*, 2013). HIGS targeting fungal CYP51 gene has been claimed to be an alternative to chemical treatments to control this devastating fungal disease. The HIGS approach is also working for the oomycete plant pathogen *Phytophthora capsci* (Vega-Arreguin *et al.*, 2014). *In planta* expression of hairpin construct against the *PcAvr3a1*gene leads to down regulation of this gene in *P. capsici* allowing infection in non-host tobacco plant. The *Phytophthora parasitica* did not respond to the applied HIGS approach (Zhang *et al.*, 2011). This was explained by lack of proper machinery required for the uptake of silencing signals in the oomycete pathogen, which is in contrary to what is already a well-established methodology in oomycete research. It was shown beyond doubt that oomycetes do have the machinery needed for uptake of silencing molecules (Whisson *et al*., 2005; Vetukuri *et al*., 2011a).

In our work we attempted to demonstrate whether a HIGS approach could be adopted to improve the defense levels in potato against *P. infestans* (Jahan *et al.* 2015). First, in order to generate a proof of concept, a hairpin RNA (hp-RNA) construct was designed against the *GFP* marker gene in *P. infestans* via potato, which generated a 55-fold reduction in signal intensity. A reduction of the GFP at protein level was also shown by Western blot in the transgenic potato lines compared to wild-type plants upon infection with the *GFP*-tagged *P. infestans* isolate. On the basis of the GFP analyses, three *P. infestans* genes: *PiGPB1 (*G-protein ß-subunit), known to be important for sporangia formation through phosphatase-mediated signaling in mycelia (Kasahara and Nuss, 1997; Latijnhouwers *et al.*, 2003); *PiCESA2* (cellulose synthase A2), associated with appressoria formation (Grenville-Briggs *et al.*, 2008) and *PiPEC* (pectinesterase), essential for successful establishment of infection (Ospina-Giraldo *et al.*, 2010), together with *PiGAPDH* (glyceraldehyde 3-phosphate dehydrogenase) (Zeng *et al.*, 2006) involved in basic cell maintenance were evaluated. Out of these genes, the hpRNA construct targeting the G protein ß-

subunit (*PiGPB1*) resulted in the most restricted disease progress. *PiGPB1* is a single copy gene and we found fewer and defective sporangia formation upon silencing of this gene in *P. infestans*

Silencing of *PiCESA2, PiPEC* and *PiGAPDH* could not inhibit *P. infestans* colonization consistently. This could be due to gene redundancy. *CESA*, *PEC* and *GAPDH* belong to gene families of varying size in *P. infestans,* making it challenging to design optimal hp-gene constructs. However, the work demonstrates that a host-induced gene silencing approach is functional against *P. infestans* but is highly dependent on choice of target gene for a successful outcome.

In order to confirm that the observed phenotype was due to the presence of sRNAs homologous to hpRNA constructs in transgenic plants, Northern blot hybridizations were employed. Probes specific to *PiGPB1* detected antisense sRNAs, ranging from 21, 25, 26, 27 and 28 nt in size. Illumina sequencing of sRNAs generated from wild type and *hp-PiGPB1* transgenic plants resulted in unique but few 24/25 nt size class sRNAs only mapping to the *PiGPB1* gene in the transgenic plants at 24, 48 and 72 hpi not seen in control plants. HIGS or RNAi turns out to be an additional strategy to be adopted in potato as a complement to resistance gene deployment to reduce disease progress by *P. infestans*. The schematic representation of HIGS approach between potato and *P. infestans* is shown in Fig. 5.

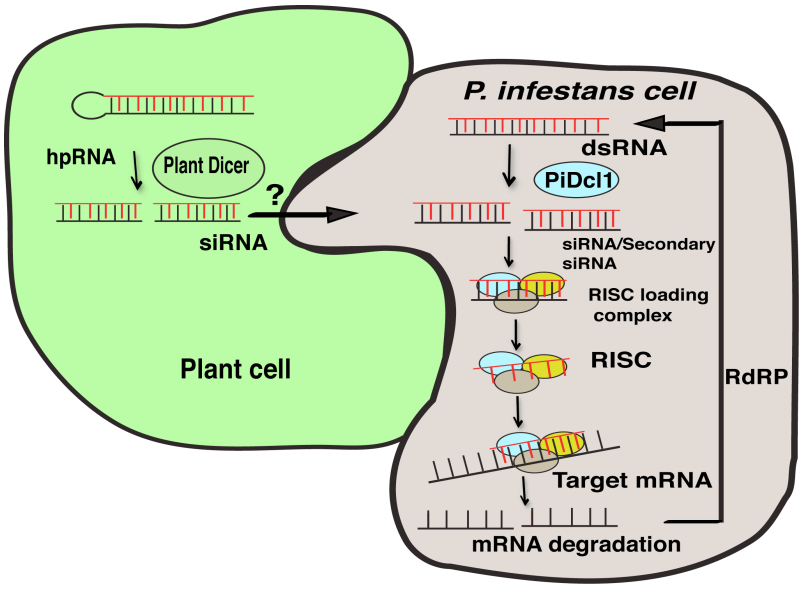

Figure 5. Schematic model of the HIGS process between potato and *P. infestans*. Hairpin RNA construct targeting gene of interest in *P. infestans* is expressed in potato, which is processed by plant Dicer or Dicer-like protein into short double stranded siRNA transported by an unknown mechanism to *P. infestans* during infection. The siRNA duplex gets incorporated into RISC loading complex. The duplex is unwound and the antisense strand incorporated to an Argonaute (Ago) protein, which then binds to homologous mRNA and degrades it or inhibits its translation. RNA dependent RNA polymerase uses degraded RNA as template to generate double-stranded RNA and causes amplification of the silencing signal through the generation of secondary siRNA.

We do not know much about the mechanism of potato-*P. infestans* trans-kingdom transfer of siRNAs. It was shown that the fungus *Botrytis cinerea* delivers sRNAs to *A. thaliana* and hijacks the host RNAi machinery and selectively silences host immunity genes during the infection process (Weiberg *et al.*, 2013). For the transport of non-vesicular extracellular sRNA in *C. elegans,* SID-1 and SID-2 proteins have been identified (Winston *et al.*, 2002; Winston *et al.*, 2007). SID-2 receptor-mediated endocytosis is causing the internalization of dsRNA from the intestinal lumen and SID-1 is helping the dsRNA to escape from endosome into the cytoplasm. Any SID homologs are not found in *P. infestans* or in potato. In humans, exosomes are suggested to be involved in transport of sRNAs where they remain protected (Valadi *et al.*, 2007) and form complexes with Ago2 (Turchinovich *et al.*, 2011). Similarly,

exosomes are thought be involved in the barley-*Blumeria graminis* interaction (Nowara *et al.*, 2010). In *Trypanosoma cruzi* extra cellular vesicles have been shown to transfer sRNAs to the mammalian host cells and affect gene expression (Garcia-Silva *et al.*, 2014). After entering the host, the sRNAs possibly use the RNAi machineries in the host target cells. In the *B. cinerea* case, upon entering the host cell, the fungal sRNAs utilize host Ago1 to exert the silencing effect (Weiberg *et al.*, 2013).

3.3 Trans-kingdom RNAi between potato and *P. infestans* – can resistance genes be affected? (Paper IV)

R genes in plants are tightly regulated to ensure expression of R proteins only during pathogen attack (Park and Shin, 2015). Endogenous siRNAs and miRNAs are proved to be involved in this gene regulation (Katiyar-Agarwal *et al.*, 2006; Li *et al.*, 2012). For example, it was shown that two miRNAs nta-miR6019 and nta-miR6020 guide the transcript cleavage of NB-LRR type immune receptor N from tobacco, which confers resistance to tobacco mosaic virus (TMV) (Li *et al*. 2012). In our study we have used a resistant potato cultivar Sarpo Mira possessing *R3a, R3b, R4,* and the *Rpi-Smira1* genes (Rietman *et al.*, 2012). The high resistance level is now starting to breakdown. In order to decipher the molecular events underlying this resistance breakdown we have started to analyze the transcript change and the possible role of sRNAs on transcript regulation during infection with a compatible isolate (NL) of *P. infestans*. In our sRNA sequenced data, 93 potato miRNA families were predicted (Jahan *et al.* 2015). With reference to current database information four miRNAs were potato specific and 16 were conserved in different plant species. The most abundant size class of miRNAs was 21 and 22 nt long. Among the total predicted miRNAs 86% were located in the intergenic regions, 55% were found in exon sequences and 45% in intron regions. According to our transcript sequencing data 486 genes were down-regulated in Sarpo Mira infected with the aggressive isolate compared to a nonaggressive

isolate of *P. infestans* and the uninfected control. From these down-regulated genes, 21 were predicted to be targeted by potato miRNAs. Targeted degradation of candidate genes by the predicted miRNAs was validated by an ETPemiR approach. The expression analysis of pri and mature miRNA was checked by qRT-PCR and by stem-loop qRT-PCR respectively. In plants the Dicer-like protein 1 (DCL1) is responsible for conversion of pri-miRNA into mature miRNA (Kurihara and Watanabe, 2004). Recently it has been shown that *Phytophthora* impairs miRNA-mediated immunity in plants by deregulating the processing of pri-miRNA to mature miRNA (Qiao *et al.*, 2015). Therefore, we speculate that *P. infestans* is deregulating DCL1 mediated processing of our candidate pri-miR to mature miRNA. Several components are most likely involved in this process and near future analysis will help us to dissect and enhance our understanding of how *P. infestans* uses sRNAs to evade plant immunity responses.

4 Conclusions

These are the main conclusions from the works presented in this thesis.

- sRNAs of 21 nt and 25 nt dominate the sRNA population in *P. infestans*. sRNAs mapped to CRN, RXLR and mostly to TEs.
- Differential abundance of sRNAs mapping to some effector genes between R0 and 3928A isolate of *P. infestans* indicates the link between sRNAs and variability in pathogenicity.
- Silencing of Dicer and Argonaute genes provides evidence that biogenesis of 21 nt sRNAs is Dicer-dependent and accumulation of longer sRNAs is influenced by silencing of Argonaute genes.
- The majority of tRFs in *P. infestans* are 25-30 nt long and generated through cleavage of tRNA anticodon loop area.
- PiAgo1 and PiAgo4 seem to be associated with accumulation of tRFs.
- Differential accumulation of some specific tRFs is observed during the infection process indicating their possible involvement in infection.
- A HIGS approach could be adopted to improve the defense levels in potato against *P. infestans* and choice of target gene is crucial for a successful outcome.
- 93 potato miRNA families are predicted in resistant cultivar Sarpo Mira infected with the compatible isolate NL.
- The most abundant size class of miRNAs are 21 and 22 nt long and

the majority of predicted miRNAs are located in the intergenic region.

- In NL infected Sarpo Mira, down regulated resistance genes are targeted by its own miRNAs providing clues about pathogen-mediated regulation of host resistance genes by endogenous miRNAs.

5 Future perspectives

This thesis work holds great importance in advancement of our knowledge about potato-*P. infestans* interaction. However this work suggests further exploration on some areas.

The host-induced gene silencing of *P. infestans* clearly contributes to reduce disease progress in potato and should be regarded as an additional strategy to complement resistance gene deployment. It would be interesting to test if broader resistance could be achieved by combining several RNAi transgenes, preferably targeting single copy genes of importance in the infection process.

The mechanism of sRNA transport between host and pathogen is still not established. Understanding potential sRNA transport from *P. infestans* to the host plant hopefully will open up alternative revenues to control this important plant pathogen. It would be very exciting although not easy to study the sRNA transport mechanism in the potato-*P. infestans* pathosystem. Transport proteins with RNA binding motifs could be potential candidates to be investigated as transporter of sRNAs. Exosomes are another possible candidate to be responsible for sRNA transport. Isolation of exosome from the phloem of *P. infestans* infected potato plant followed by extraction of sRNA and subsequent sequencing could reveal if the exosomes are carrying the sRNAs or not.

From the sRNA sequencing data on potato-*P. infestans* interaction samples, siRNA population in potato and their target transcripts both in potato and *P. infestans* need to be clarified. This would aid in exploring the impact of siRNA-mediated regulation of specific gene in potato-*P. infestans* interaction.

CRISPR/Cas9 is a promising and highly efficient technique for gene editing. It would be worth trying to apply this technique for gene editing both in potato and *P. infestans*.

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