The role of the RNA silencing network on the co-evolution of *Phytophthora infestans* and *Solanum* spp.

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Statement of authorship

I hereby declare that this dissertation is the result of my own work. No other person's work has been used without due acknowledgement. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

Sophie de Vries

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1 Summary

RNA silencing is an important regulatory system in plants, controlling everything from development to abiotic and biotic stress responses. Negative post-transcriptional regulation of gene expression through small RNAs (sRNA) enables the plant to rapidly respond to pathogens and fine-tune these responses. Recent evidence indicates that several classes of pathogens trigger shifts in host sRNA production and produce RNA silencing suppressors to target the host's silencing machinery. What is more, the plants' own microRNAs (miRNAs) were shown to regulate plant resistance (*R*) genes. The work presented here concentrates on how the RNA silencing network in tomatoes (*Solanum* spp.) is shaped by and also contributes to the co-evolution of the pathogen *Phytophthora infestans* with its hosts.

In the Solanaceae, the miRNA family miR482/2118 regulates *R* gene expression. We identified eight different family members of miR482/2118 in the genomes of 14 species of Solanaceae. Of these eight, seven are conserved in the wild tomatoes. Analyses of the evolutionary rates of the precursors of these eight types showed that each family member evolves under a different evolutionary constraint. In particular, the evolutionary rate of the mature miRNA region was elevated in four of the eight miR482/2118 family members. This points to lineage-specific evolution of the miR482/2118 family, potentially related to the *R* gene repertoire of the Solanaceae species. The investigation of the evolution of the miR482/2118 – *R* gene targeting network showed that a comparable percentage of *R* genes is targeted in tomato and potato. This indicates that the miRNA – *R* gene targeting network is robust. Additionally, *R* gene duplication is likely supported by miRNA targeting. This robust network is, nevertheless, evolutionary dynamic: orthologous and paralogous *R* genes are often targeted by a different miR482/2118 member rather than the same. Together this suggests that the adaptability of this network might not only allow for but could even promote *R* gene evolution. miRNA regulation of *R* genes may therefore facilitate rapid adaptation of a plant host to a pathogen and may therefore be advantageous for the hosts.

Plants infected by pathogens that produce RNA silencing suppressors reveal a down-regulation of miR482/2118 members. miR428/2118 down-regulation results in elevated *R* gene expression, suggesting an additional role of miR482/2118 as a counter-counter defense of plants against pathogen RNA silencing suppressors: suppression of RNA silencing includes the reduction of miR482/2118 levels and consequently leads to an up-regulation of *R* genes and therefore enhances immunity. Yet, RNA silencing suppressors enhance virulence of pathogens. This seems contradictory. Therefore, to better understand the role of RNA silencing suppressors in the RNA silencing network, we analyzed the one known RNA silencing suppressor in *P. infestans*, *Phytophthora* Suppressor of

RNA silencing 2 (*Pi*PSR2). Homologs of Pi*PSR2* are present in many *Phytophthora* species, underpinning the importance of PSR2 for the virulence of many economically important *Phytophthora* species. We show that Pi*PSR2* evolves under purifying selection and that higher evolutionary rates of PSR2 proteins were associated with the host range of different *Phytophthora* pathogens, suggesting that host range is an important factor in the evolution of *PSR2*. PSR2 inhibits the production of a certain type of phased secondary small interfering RNA (phasiRNA), including those phasiRNAs that are triggered through the action of miR482/2118. Therefore, PSR2 is a key link to understanding the evolution of counter-counter defense. We discovered that Pi*PSR2* is either up-regulated during the biotrophic phase or constantly expressed throughout infections in wild and cultivated tomatoes, depending on the *P. infestans* isolate and the tomato host. A high expression was associated with lower virulence, suggesting the necessity of a tight Pi*PSR2* expressional regulation. Based on these results, I concluded that PSR2 may globally shift phasiRNA production in *Phytophthora* hosts and that the negative effects from a de-repression of *R* genes by phasiRNAs are counterbalanced by local shifts in phasiRNA regulation of development and nutrient signaling.

2 Zusammenfassung

RNA-Silencing ist ein wichtiger Mechanismus, um die Entwicklung von Pflanzen und ihre Reaktionen auf äußere Stimuli, wie beispielsweise Pathogene, zu regulieren. Es ist daher nicht verwunderlich, dass die Produktion von pflanzlichen kleinen RNAs global durch eine Infektion verändert wird und dass Pflanzenpathogene Suppressoren für den pflanzlichen RNA-Silencing-Apparat besitzen. Zusätzlich besitzen Pflanzen microRNAs (miRNAs), welche die Transkriptmenge von Resistenzgenen (*R*-Gene) reduzieren. All dies deutet darauf hin, dass das RNA-Silencing-Netzwerk eine entscheidende Rolle in der Interkation von Pflanzen und Pathogenen spielt. Diese Arbeit beschäftigt sich mit der Rolle des RNA-Silencing-Netzwerks in der Koevolution von Tomaten (*Solanum* spp.) und *Phytophthora infestans*.

In den Solanaceae wird die Expression von *R*-Genen durch die miRNA-Familie miR482/2118 reguliert. In dieser Arbeit wurden insgesamt acht verschiedene Mitglieder der miR482/2118-Genfamilie in 14 analysierten Solanaceae-Genomen identifiziert. Von diesen acht verschiedenen miR482/2118-Genen sind sieben in den Wildtomaten konserviert. Die Evolutionsraten der acht miR482/2118-Precursorsequenzen sind unterschiedlich, was zeigt dass diese acht Gene unterschiedlich schnell evolvieren. Die Region in den Precursorsequenzen, welche die aktive miRNA kodiert, wies in vier von insgesamt acht miR482/2118-Precursorsequenzen eine erhöhte Evolutionsrate auf. Dies deutet auf eine artspezifische Evolution der miR482/2118-Gene hin, welche vermutlich von der *R*-Genkomposition der jeweiligen Art abhängig ist. Im Einklang mit diesem Ergebnis zeigte die Analyse des miRNA regulatorischen Netzwerks der *R*-Gene, dass immer ein vergleichbarer Prozentsatz an *R*-Genen durch die miR482/2118-Genfamilie reguliert wird. Dies deutet auf eine gewisse Robustheit des miR482/2118-*R*-Gen-Netzwerks hin. Des Weiteren lassen unsere Ergebnisse vermuten, dass die miRNA-Regulation der *R*-Gene deren Genduplikationen begünstigt. Dieses robuste Netzwerk weist zusätzlich eine dynamische Komponente auf: orthologe und paraloge *R*-Gene werden häufig von unterschiedlichen miRNAs reguliert. Es ist daher möglich, dass *R*-Gen-regulierende miRNAs eine schnelle Adaption der Pflanzen an ihre Pathogene ermöglichen.

Pflanzen, die mit Pathogenen infiziert wurden, welche RNA-Silencing-Suppressoren produzieren, zeigen reduzierte miR482/2118-Mengen. Diese Reduktion der miR482/2118-Menge führt zu erhöhten *R*-Gentranskriptmengen, was eine erhöhte Resistenz gegen Pathogene zur Folge hat. Daher wird vermutet, dass die miR482/2118-Genfamilie, zusätzlich zu ihrem Einfluss auf die *R*-Gen-Evolution, auch eine Gegenantwort der Pflanze auf die RNA-Silencing-Suppressoren ist. Dieses Konzept beinhaltet, dass eine befallene Pflanze deren RNA-Silencing-Apparat von RNA-Silencing-Suppressoren eines Pathogens attackiert wird, eine automatische Hochregulation der *R*-Gene erfährt,

da die Produktion der *R*-Gen-regulierenden miRNAs inihibiert wurde. Nichtsdestotrotz erhöhen RNA-Silencing-Suppressoren die Virulenz von Pathogenen. Dies scheint widersprüchlich. Um die Rolle von RNA-Silencing-Suppressoren in diesem Netzwerk besser zu verstehen, analysierten wir den derzeit einzigen aus *P. infestans* bekannten RNA-Silencing-Suppressor: Phytophthora *Suppressor of RNA Silencing 2* (PSR2). *PSR2* wurde in mehreren *Phytophthora*-Spezies gefunden, wodurch die Relevanz dieses Gens für die Virulenz von *Phytophthora* spp. hervorgehoben wird. Pi*PSR2* weist Anzeichen von *purifying selection* auf und eine erhöhte Evolutionsrate des PSR2-Proteins korrelierte mit der Anzahl an Wirten, die von der jeweiligen *Phytophthora*-Spezies befallen werden können. Dies lässt vermuten, dass die Fähigkeit mehrere Wirte zu infizieren, die Evolution von *PSR2* stark beeinflusst. PSR2 inhibiert die Produktion sogenannter *phased secondary small interfering RNAs* (phasiRNAs). miR482/2118 ist in der Lage die Produktion solcher phasiRNAs zu initiieren. PSR2 ist daher eine wichtige Schnittstelle um die Rolle von *R*-Gen-regulierenden miRNAs und phasiRNAs besser zu verstehen. In dieser Arbeit wurde gezeigt, dass Pi*PSR2* entweder in der biotrophen Phase der Infektion hochreguliert oder konstant während der gesamten Infektion exprimiert wid. Eine höhere Pi*PSR2* Expression ist dabei mit einer geringen Virulenz von *P. infestans* assoziiert. Dies deutet auf die Notwendigkeit einer exakten Regulation der Pi*PSR2*-Menge während einer Infektion hin. Es ist daher plausibel, dass PSR2 ein globaler Regulator von phasiRNAs in den verschiedenen *Phytophthora* Wirten ist. Der Einfluss von PSR2 auf die *R*-Gentranskriptmenge, welche durch die Suppression des RNA-Silencings erhöht ist, wird dadurch ausgeglichen, dass die Mengen von entwicklungs- und nährstoffaufnahmeregulierenden phasiRNAs zur gleichen Zeit reduziert werden.

3 List of publications

3.1 Publications included in this thesis

1.) **de Vries, S.**, Kloesges, T., and Rose, L.E. (2015). Evolutionarily dynamic, but robust, targeting of resistance genes by the miR482/2118 gene family in the Solanaceae. Genome Biol. Evol. *7*, 3307-3321.

2.) **de Vries, S.**, von Dahlen, J.K., Uhlmann, C., Schnake, A., Kloesges, T., and Rose, L.E. Signatures of selection and host adapted gene expression of the *Phytophthora infestans* RNA-silencing suppressor PSR2. Mol. Plant Pathol. under review.

3.2 Additional publications

1.) de Vries, J., Fischer, A.M., Roettger, M., **Rommel, S.***, Schluepmann, H., Bräutigam, A., Carlsbecker, A., and Gould, S.B. (2016). Cytokinin-induced promotion of root meristem size in the fern *Azolla* supports a shoot-like origin of euphyllophyte roots. New Phytol. *209*, 705 -720.

2.) **de Vries, S.**, Nemesio-Gorriz, M., Blair, P.B., Karlsson, M., Mukhtar, M.S., and Elfstrand, M. (2015). Heterotrimeric G-proteins in *Picea abies* and their regulation in response to *Heterobasidion annosum s.l.* infection. BMC Plant Biol. *15*, 287.

3.) Rauch, C., de Vries, J., **Rommel, S.***, Rose, L.E., Woehle, C., Christa, G., Laetz, E.M., Waegele, H., Tielens, A.G.M., Nickelsen, J., Schumann, T., Jahns, P., and Gould, S.B. (2015). Why it is time to look beyond algal genes in photosynthetic slugs. Genome Biol. Evol. *7*, 2602-2607.

4.) Oliva, J., **Rommel, S.***, Fossdal, C.G., Hietala, A.M., Nemesio-Gorriz, M., Solheim, H., and Elfstrand, M. (2015). Transcriptional responses of Norway spruce (*Picea abies*) inner sapwood against *Heterobasidion parviporum*. Tree Physiol. *35*, 1007-1015.

*Sophie de Vries' former name was Sophie Rommel.

4 Conference contributions

4.1 Oral presentations

Rommel, S.*, Kloesges, T., and Rose, L.E. (2014). microRNA signaling in tomatoes during *Phytophthora infestans* infection. *Oomycete Molecular Genetics Network Meeting (OMGN), Norwich, UK.*

Rommel, S.*, Kloesges, T., and Rose, L.E. (2014). Resistance – shaping miRNAs in wild tomatoes. *XVIII Tomato Working Group Eucarpia Meeting, Avignon, France.*

4.2. Poster presentations

Rommel, S.*, Kloesges, T., and Rose, L.E. (2015). Differential miRNA-mediated *R* gene regulation in wild and cultivated tomato during *Phytophthora infestans* infection. *Meeting of the German Botanical Society (DBG), Munich, Germany*.

Rommel, S.*, Kloesges, T., and Rose, L.E. (2015). The evolution of plant-pathogen defense-layers, focusing on miRNA mediated negative regulation, in plant-parasite interactions. *Society of Molecular Biology and Evolution meeting (SMBE), Vienna, Austria*.

*Sophie de Vries' former name was Sophie Rommel.

5 Introduction

5.1 Plant-pathogen interactions

Of the numerous microbes a plant is exposed to, only a few are able to enter it. Once the first barriers of entrance, such as the cuticle and the cell wall (reviewed in Hückelhoven 2007 and Serrano et al. 2014), are overcome, microbes trigger the molecular plant defense system. Membrane-bound pathogen-recognition receptors (PRRs), which consist of receptor-like kinases (RLKs) and receptor-like proteins (RLPs), recognize common microbial features (Figure 1a, e.g. Goméz-Goméz and Boller 2000, Jones et al. 1994, reviewed by Zipfel 2009). These common and highly conserved microbial features are called Microbial-associated-molecular-patterns (MAMPs) and include compounds such as chitin (from the fungal cell wall, reviewed by Shibuya and Minami 2001), flagellin (a structural protein from the microbial flagella, Felix et al. 1999) and secreted peptides or proteins (e.g. Ricci et al. 1989, Lee et al. 2009). The detection of MAMPs by PRRs results in the induction of the general pathogen-triggered immunity (PTI, Goméz-Goméz and Boller 2000). The PTI response restricts pathogen entrance and growth through processes such as cell wall reinforcements, callose deposition at the point of pathogen entry or toxin production, including reactive oxygen species (ROS) (Figure 1a, Felix et al. 1999, reviewed by Shibuya and Minami 2001). In case of *Phytophthora infestans* these ROS induce a hypersensitive response (HR) and local cell death early in the infection to stop pathogen growth (Figure 1a, Vleeshouwers et al. 2000).

Three different types of pathogen life styles inside a plant have been observed: biotrophic, necrotrophic and hemibiotrophic (reviewed by Glazebrook 2005). An example for a biotrophic pathogen is *Cladosporium fulvum*, a tomato pathogen (Cooke 1883, for review see Joosten and de Wit 1999). *C. fulvum* depends on its living host; it only passes the tomato cell wall, but never invades the cytoplasm of its host and hence is strictly growing in the host's apoplast (de Wit 1977). However, other biotrophs, such as the downy mildew species *Hyaloperonospora* spp., form haustoria that invaginate the host plasma membrane (Fraymouth 1956, Chou 1970). The plasma membrane surrounding the haustoria is then modified and becomes the extra-haustorial membrane that harbors plant and pathogen trans-membrane proteins (Voegele et al. 2001, Wang et al. 2009). This way, the extra-haustorial membrane becomes the interface of the molecular cross-talk between pathogen and host cells (Voegele et al. 2001, Whisson et al. 2007). In contrast, necrotrophic pathogens, such as *Botrytis cinerea*, destroy their hosts' cells by inducing cell death and cell lysis (reviewed by van Kan 2006). They gain their nutrients from cell degradants (reviewed by van Kan 2006). Hemibiotrophic pathogens are an intermediate of the two other life style forms (reviewed by Glazebrook 2005): these

pathogens first grow biotrophically, but then switch to the necrotrophic stage at a later time point in their infection cycle. A famous example of a hemibiotrophic pathogen is the oomycete *P. infestans.*

But how do any of these pathogens hide their continued presence inside the plant? They establish their further growth by secreting molecules into the plant's apoplast and cytoplasm; these secreted molecules include toxins and (small) proteins (called effectors, Whisson et al. 2007, Andersson et al. 2010), the latter of which suppress PTI responses (Figure 1b, Fabro et al. 2011). The plant can recognize these pathogen-secreted peptides and effectors and/or their manipulation via a battery of resistance proteins (R proteins) and elicit new defense responses – hence coined effector triggered immunity (ETI) (reviewed by Cui et al. 2015). R proteins are often, but not exclusively Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (reviewed by Gururani et al. 2012). The ETI responses, triggered by R proteins, include modulation of the plant transcriptome, HR and induced cell death (Figure 1b, Eulgem et al. 2004, Armstrong et al. 2005). Yet, only if the R protein recognizes the respective effectors, is a defense response initiated (e.g. Rehmany et al. 2005, Allen et al. 2008, Krasileva et al. 2010) - without successful recognition of the pathogen by the plant, plant-induced necrosis due to cell death and HR do not occur.

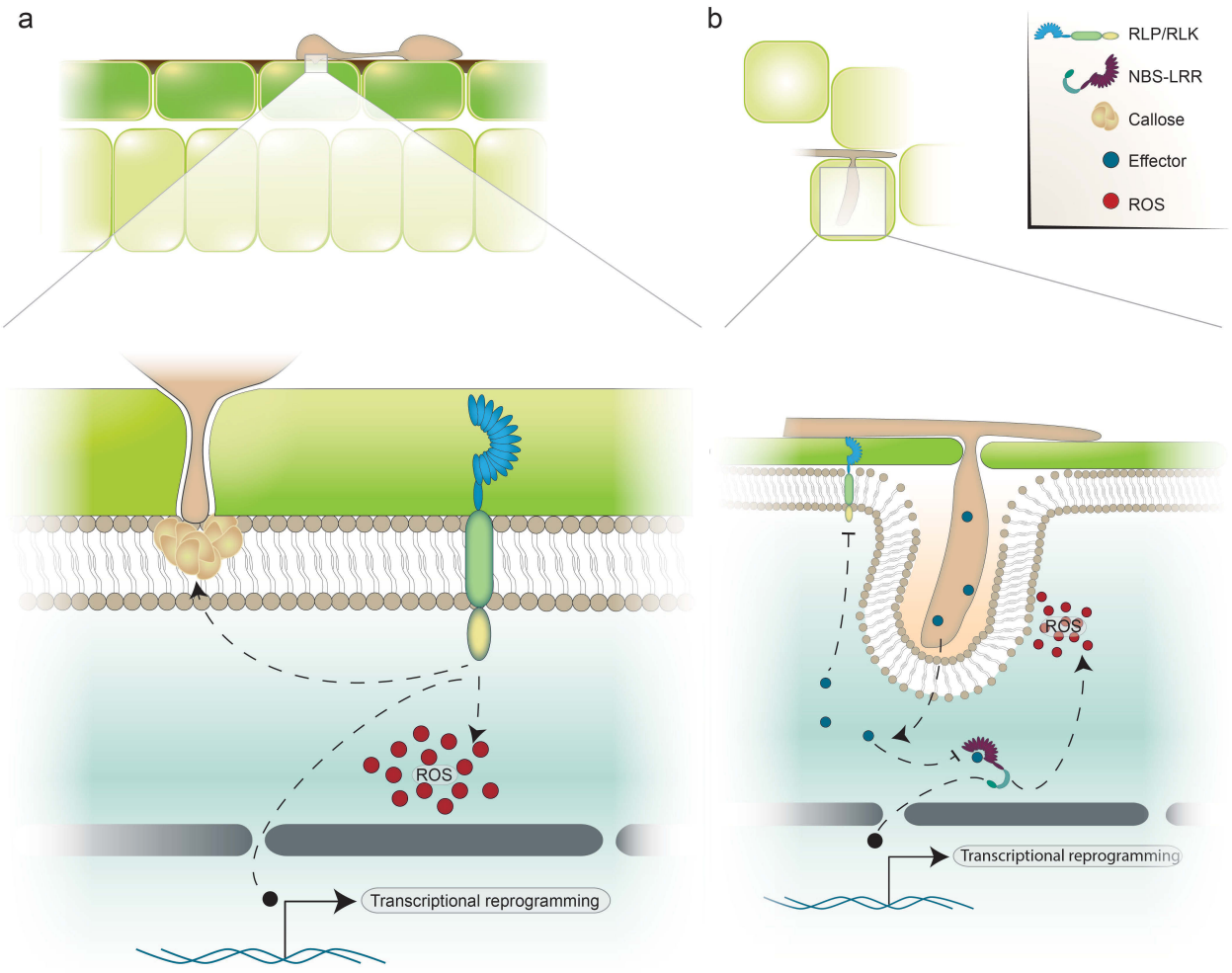

Figure 1. Plant defense responses towards pathogens. (a) Pathogens are recognized by plant receptor-like proteins or kinases (RLP/RLK) because of their conserved molecular patterns, such as flagellin or chitin. RLPs/RLKs initiate a downstream signaling, leading to a vast transcriptional reprogramming, enhanced callose production and in some cases early ROS accumulation and HR. These early defense responses elicited by MAMPs are termed PTI. (b) Pathogens can overcome the first step of plant immunity by secreting effector molecules that interfere with RLPs/RLKs to prevent defense signaling. R proteins (mainly NBS-LRRs) recognize these effector proteins (or their actions) and trigger a more pathogen-specific defense response. This response involves transcriptional reprogramming tailored to the specific pathogen and includes e.g. ROS production, HR and cell death. NBS-LRRs, in turn, can also be targeted by effectors to inhibit their function. Figure inspired by Wirthmueller et al. 2013.

5.2 The *Phytophthora infestans – Solanum* pathosystem

P. infestans is one of the most (in)famous pathogens in the world. It was due to a severe *P. infestans* outbreak in the 1840s that a great proportion of Ireland's population either emigrated or died due to starvation (Boyle and Ó Gráda 1986). *P. infestans* belongs to the oomycetes, which belong to the Stramenopiles, a group of eukaryotes that include diatoms and brown algal kelps (Baldauf et al. 2000, Adl et. al. 2012). Therefore, in contrast to fungi, oomycetes are more closely related to plants than they are to animals (Baldauf et al. 2000). The center of origin of *P. infestans* is still debated, the South American Andeans and Central Mexico being the two most likely candidates (Gómez-Alpizar et al. 2007, Goss et al. 2014, Martin et al. 2016). From there, *P. infestans* spread and by now has a global distribution coinciding with its cultivated host species tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) (Yoshida et al. 2013). The pathogen's host range is much broader, though, as it is able to naturally infect wild tomato and potato species (Adler et al. 2002, Flier et al. 2003, Garry et al. 2005).

The center of origin for wild tomatoes (section *Lycopersicon*) was proposed to be the Andean regions in South America (Jenkins 1948). The highest degree of species richness of wild *Solanum* (section *Petota*) is found in Central and South America (Hijmans and Spooner 2001). Hence many studies investigate wild tomatoes and potatoes as a potential genetic resource for resistance genes against *P. infestans* (e.g. Moreau et al. 1998, van der Vossen et al. 2003, Lokossou et al. 2010, reviewed by Vleeshouwers et al. 2011, reviewed by Nowicki et al. 2012). Many introgressed *R* genes in cultivated potato and tomato were identified from *Solanum* species of the section *Petota* from different areas of Central Mexico (reviewed by Vleeshouwers et al. 2011). But *R* genes from wild tomatoes were also introgressed into the cultivated crops (Lin et al. 2014). Despite the in-depth searches for new *R* genes, resistance against *P. infestans* in tomato and potato is often not durable (reviewed by Vleeshouwers et al. 2011). In the pursuit of durable resistance, many avenues are explored, foremost the study of (molecular) resistance mechanisms within the host species (e.g. Vleeshouwers et al. 2000, Engelhardt et al. 2012, McLellan et al. 2013), but also the detailed analyses of the *P. infestans* biology (cf. Fry et al. 2015).

P. infestans can propagate asexually and sexually. While the asexual spores are motile and associated with spreading the disease over longer distances (reviewed by Judelson and Blanco 2005), the sexual spores are viable for a long time under natural conditions (Drenth et al. 1995) and provide *P. infestans* with the ability to recombine (Tooley et al. 1985) hence resulting in the ability to constantly re-invent and adapt its effector repertoire. *P. infestans'* asexual life cycle during infection of a host plant has been extensively investigated (Figure 2): Motile zoospores land on leave surfaces,

or alternatively on tomato roots or potato tubers, where they encyst and attach to the cell surface (reviewed by Judelson and Blanco 2005). A germination tube grows out of the zoospore and forms an appressorium (reviewed by Judelson and Blanco 2005). Appressoria apply pressure on the host cell surface (Howard et al. 1991) and thus enable invasion of *P. infestans* into the apoplastic space of the host. In its biotrophic phase *P. infestans* forms finger-shaped haustoria (Blackwell 1953, Allen and Friend 1983). These haustoria are used to secrete for example effectors into the host's cytoplasm (Whisson et al. 2007), making it the interface for plant-pathogen communication. After a few days, *P. infestans* transits into a necrotrophic phase (e.g. Cooke et al. 2012, Zuluaga et al. 2016a). During this phase, sporangiophores with sporangia at their tips are formed (Farlow 1883).

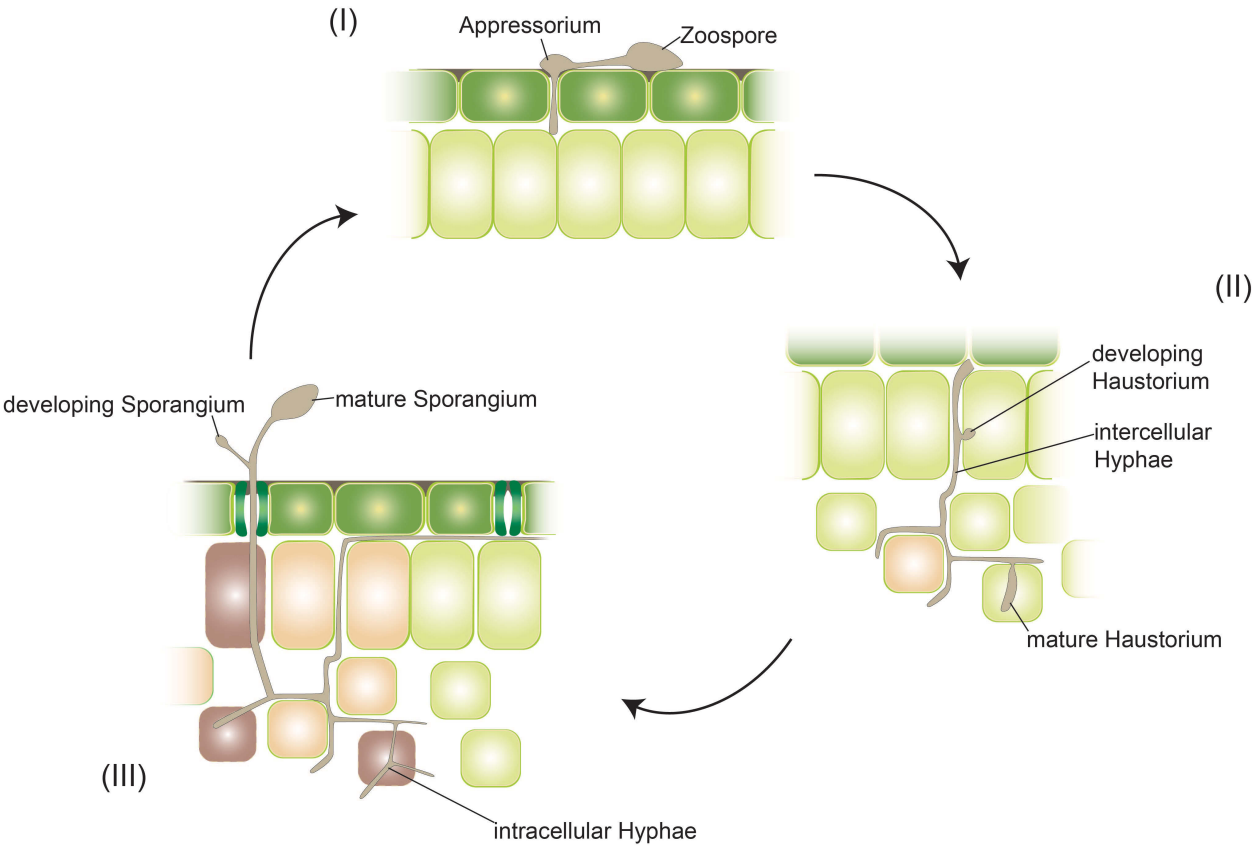

Figure 2. Asexual life cycle of *P. infestans* during infection. (I) *P. infestans* zoospores land on or move towards e.g. a leaf surface where they encyst. A germination tube grows from the encysted zoospore and forms an appressorium on its tip. The pressure the appressorium applies on the plant cell surface enables *P. infestans* to enter the apoplastic space of its host. (II) Hyphae grow through the plant's apoplast. Early on in the infection, bud-shaped haustoria are formed (developing haustoria), which penetrate through the plant cell walls (however never disrupting the plant plasma membrane). These developing haustoria mature into finger-like shaped haustoria. Plant responses, such as HR, are initiated (beige cells). (III) In the end of the *P. infestans* infection, hyphae invade the plant cells and induce cell death (brown cells). Sporangiophores are formed, which grow out of natural openings (such as stomata), were sporangia develop. Under the right conditions (cold and wet) the sporangia open and release the motile zoospores. Figure inspired by Judelson and Blanco 2005.

The distinct life cycle stages of *P. infestans* and its close relatives are characterized by extensive transcriptional remodeling both when the different physiological structures are induced in the lab and also *in planta* during infection: The study of Judelson and colleagues (2008) indicates that most genes required during *P. infestans*' biotrophic life phase as well as for many pathogen responses (e.g. defense proteins against ROS) are already expressed in germinating cysts. This is in agreement with a study of a closely related pathogen *Phytophthora capsici* that suggests strong modulation of tomato PTI in the first eight hours of infection by pathogen effector molecules (Jupe et al. 2013). *Phytophthora sojae*, another close relative, also shows a specific effector repertoire expressed during the early biotrophic phase (Wang et al. 2011). Oh and colleagues (2009) showed that effector expression in *P. infestans* mycelium is partially distinct from effector expression during tomato infection. Furthermore, that study points towards a temporal component in effector expression during *P. infestans* infections. This was recently supported by global gene expression analyses of a *P. infestans* infection in tomato sampled at different time points after inoculation (Zuluaga et al. 2016a).

Besides global gene expression studies that aim to understand the biology of *P. infestans* and its relatives, studies focusing on specific (effector) genes showed that there is a tight regulation of *P. infestans* infection and the corresponding plant defense reactions: *P. infestans* has two types of effector families, RxLR and Crinkler (CRN) proteins (Torto et al. 2003, Rehmany et al. 2005). One RxLR effector, Suppressor of necrosis 1 (SNE1) suppresses *P. infestans*-mediated (through its Nep1-like protein (*Pi*NPP1)) and host-mediated cell death (through effector – R protein recognition) (Kelley et al. 2010). Moreover, *SNE1* and Pi*NPP1* expression levels are mainly antagonistic: *SNE1* has a higher expression in the biotrophic phase, while Pi*NPP1* genes show increased expression during the necrotrophic phase (Kelley et al. 2010). Likewise, patterns in host expression profiles are equally dependent on the pathogen and its life stage – either because they are pathogen manipulated (e.g. Kelley et al. 2010, McLellan et al. 2013) or because the plant itself adjusts to the stressor (e.g. McLellan et al. 2013). Zuluaga et al. (2016b) showed that the response of *S. lycopersicum* towards *P. infestans* is also tightly linked to the life cycle stages of *P. infestans*: The authors observed that the biotrophic phase triggered differential regulation of the primary metabolism and an up-regulation of stress-related signaling in *S. lycopersicum*. They also found that the transition led to a down-regulation of genes relevant for plant immunity, including some coding for proteins that are involved in ROS generation. In contrast, in the necrotrophic phase that study observed up-regulation of cell-death related genes. This supports that *P. infestans* is able to manipulate its host's cell death induction. In the model Solanaceae *Nicotiana benthamiana,* several plant transcription factors were up-regulated in response to *P. infestans'* exudates and MAMPs, while they were down-regulated in

response to the pathogen (McLellan et al. 2013). Moreover, McLellan and colleagues (2013) showed that a specific RxLR effector of *P. infestans* is able to inhibit the release of NAC transcription factors from the ER, prohibiting their re-localization to the nucleus. NAC transcription factors are associated with defense responses in *Arabidopsis thaliana* (Bu et al. 2008, Kim et al. 2012). Additionally, several CRN effectors have been shown to be targeted to and able to enter the *N. benthamiana* nucleus (Schornack et al. 2010). All this supports, that the *Solanum* spp. hosts dynamically react to *P. infestans* infections via transcriptional remodeling, followed by a manipulation of the transcriptional responses by the pathogen, leading to a mixture of plant and pathogen determined transcription.

5.3 Evolution of *R* and effector genes

Host-pathogen interactions require a fine-tuned molecular communication of both partners. Often these involve protein-protein interactions of pathogen effectors with host proteins: The host proteins are either R proteins or other proteins that are guarded by R proteins (Jia et al. 2000, Mackey et al. 2002, Mackey et al. 2003, Axtell and Staskawicz 2003, Krasileva et al. 2010). Such protein-protein interactions lead to effector detection and plant defense responses (Krasileva et al. 2010). It is hence conceivable that pathogens need to avoid effector detection to increase their virulence. Therefore, one of the observed effects of host-pathogen co-evolution is an enhanced amount of amino acid (aa) changes in proteins that are involved in this interactions (see Stuckenbrock and McDonald 2009 for review). This suggests that natural selection acts on both, effector and *R* genes. Two scenarios have been observed so far: In the first scenario, effector and corresponding *R* genes maintain many non-synonymous polymorphic alleles at intermediate frequencies within the analyzed species (e.g. Allen et al. 2004, Bakker et al. 2006, Rose et al. 2007). Here, every R protein allele will only detect a subset of the effector alleles, and some effectors may even not be detected at all by the available R proteins (e.g. Allen et al. 2008, Hall et al. 2009). This type of selection is called balancing selection. In the second scenario, a major effector allele is present in the pathogen and a major *R* gene allele is present in the host (Gout et al. 2007, Huang et al. 2008). Accordingly it is hypothesized, that if the R protein detects this effector variant, this variant will be lost from the pathogen and a new, at that time point non-detectable allele will increase in frequency over time until fixation (cf. Lo Presti et al. 2015). If so, this type of selection will promote non-synonymous divergence between different species and is called a selective sweep. Many oomycete effector genes are subjected to balancing selection, among them the *Hyaloperonospora arabidopsidis ATR1* and *ATR13* and the *P. infestans Avrblb2* (Allen et al. 2004, Rehmany et al. 2005, Allen et al. 2008, Oliva et al. 2015). The corresponding *R* genes to *ATR13* and *ATR1* from *A. thaliana*, namely *RPP1* and *RPP13*, show the same pattern as the effector genes within species (Botella et al. 1998, Rose et al. 2004). The corresponding *R* gene to *Avrblb2, Rpi-blb2,*

however seems to be a recent invention, with no aa substitutions, which is in agreement with the observation that so far only one Avrblb2 variant was able to overcome the resistance derived from Rpi-blb2 (Lokossou et al. 2010, Oliva et al. 2015). An example of a selective sweep has been found in the fungal effector gene *AvrLm1* of *Leptosphaeria maculans* (Gout et al. 2007). This selective sweep was hypothesized to either result from selective pressure on a genetically linked effector gene, a bottleneck or the use of the corresponding *R* gene as a major gene in resistance breeding (Gout et al. 2007).

Besides allelic variation, co-evolution has also resulted in other mechanisms that effect R and effector protein interaction. Both *R* and effector genes belong to large gene families in the genomes of plants and their pathogens (Meyers et al. 1999, Tyler et al. 2006, Haas et al. 2009). Orthology assessments have identified increased gain and loss rates in the evolution of these gene families, suggesting adaptive evolution by a presence/absence mechanism (e.g. Win et al. 2007, Haas et al. 2009, Gilroy et al. 2011). Another emerging area is differential expression of effector genes to avoid resistance responses due to R protein detection. Wang et al. (2011) found that aberrant effector expression decreases virulence of *P. sojae* on soybean. Moreover, isolates that express Pi*Avr2* are detected by the R2 protein, which confers resistance against *P. infestans*; its detection is prohibited by the expression of a Pi*Avr2-like* variant instead of the Pi*Avr2* gene (Gilroy et al. 2011). Also, Qutob and colleagues (2013) showed that transgenerational gene silencing of the Ps*Avr3a* transcript occurred in both the F_1 and F_2 progeny of a cross between an avirulent and a virulent *P. sojae* strain. This led to the conversion of all strains, even those carrying the avirulent Ps*Avr3a* allele, into virulent strains (Qutob et al. 2013). These studies show that expressional variation contributes to variation in virulence and resistance as much as sequence polymorphisms and presence/absence evolution.

5.4 RNA silencing as a new player in plant-pathogen interactions

A new player in general plant immunity is RNA silencing. RNA silencing circumscribes the production of small RNAs (sRNAs, 21 to 24 nt in length) that are produced and guided to complementary RNA or DNA sequences (reviewed by Rogers and Chen 2013). The binding of these sequences by sRNAs leads to regulatory effects on either the RNA or DNA target (reviewed by Rogers and Chen 2013). The bound RNA-target will either be degraded or the translation of the bound RNA will be inhibited, binding to DNA leads to changes in the DNA methylation patterns resulting in a transcriptional regulation (reviewed by Rogers and Chen 2013). While the manipulation of the RNA silencing machinery was long known from plant-viral interactions (Lindbo et al. 1993), it only became apparent in the last decade, that this regulatory machinery is also hijacked by other pathogens such as bacteria and oomycetes (Navarro et al. 2008, Qiao et al. 2013). The central components of the RNA silencing

machinery are Dicer-like proteins (DCLs), Argonaute proteins (AGOs) and RNA-dependent RNA polymerases (RDRs, reviewed by Bologna and Voinnet 2014). DCLs execute the first steps in sRNA biogenesis (Bernstein et al. 2001); their substrates are either primary microRNA (pri-miRNA) transcripts that form double stranded RNA (dsRNA), hairpin structures or simply dsRNA structures derived exogenously (e.g. from viruses) or endogenously (Kurihara and Watanabe 2004, Yoshikawa et al. 2005). The DCLs process these dsRNA structures into smaller dsRNA pieces: The pri-miRNA is cleaved first into a smaller hairpin structure, the precursor miRNA (pre-miRNA), and then into the functional, mature miRNA (Kurihara and Watanabe 2004). Non-hairpin dsRNAs, however, are directly degraded into the functional small interfering RNAs (siRNAs) (Yoshikawa et al. 2005). sRNAs are incorporated into AGO proteins, which direct the sRNAs to their targets and initiate the cleavage of the target sequences (Fagard et al. 2000, reviewed by Rogers and Chen 2013). In several cases, miRNA binding to the target recruits RDR6 to the mRNA (Axtell et al. 2006, Chen et al. 2010, Cuperus et al. 2010). RDR6 creates a dsRNA, which is then cleaved into sRNAs in phased 21 nt patterns (phased secondary siRNAs; phasiRNAs) (Allen et al. 2005, Yoshikawa et al. 2005).

Many components of the RNA silencing machinery are involved in the regulation of plant resistance. For example, in tomato the *Ty-1/Ty-3* resistance gene, giving partial resistance against tomato yellow leaf curl virus (Fargette et al. 1996), has been characterized to encode an RDR (Verlaan et al. 2013). A study by Ellendorf et al. (2009) palpably illustrates the complexity of sRNA silencing in plant immunity. In that study, several components of the sRNA silencing machinery either enhanced or reduced susceptibility of *A. thaliana* towards the fungal pathogen *Verticilium dahliae*. The responses were tested in infections with other pathogens and showed similar outcomes with different *Verticilium* species, but not with species of other fungal genera (Ellendorf et al. 2009). Additionally, several miRNAs have been detected that regulate NBS-LRR encoding genes in gymnosperms and dicots (e.g. Li et al. 2012, Shivaprasad et al. 2012, Boccara et al. 2014, Xia et al. 2015). One of the best-studied *NBS-LRR* targeting miRNA families is miR482/2118, which has extensively expanded in the Solanaceae and Fabaceae (Zhai et al. 2011). Their expression was shown to be down-regulated upon infection of tomatoes with *P. syringae* and viruses (Shivaprasad et al. 2012). miR482/2118 down-regulation results in enhanced *R* transcript levels and enhanced immunity (e.g. Shivaprasad et al. 2012, Boccara et al. 2014). Based on this, miR482/2118 down-regulation was interpreted as a counter-counter defense to global RNA silencing suppression by these pathogens (Shivaprasad et al. 2012).

Similar to *NBS-LRR*-suppressing miRNAs, three studies found that *P. infestans* sRNAs derive from the pathogen's own effector proteins, suggesting a potential fine-tuning of their expression during the pathogen's life cycle using the sRNA pathway (Vetukuri et al. 2012, Fahlgren et al. 2013,

Åsman et al. 2016). In addition to the differential regulation of sRNAs within host and pathogen during their interactions, pathogens use sRNAs themselves to hijack the host RNA biogenesis machinery: *B. cinerea* produces sRNAs that are translocated into the plant cell and then use the plant's AGO complex to target host genes associated with resistance responses (Weiberg et al. 2013). In conclusion, sRNAs and the sRNA biogenesis were found to be central components in plant resistance responses as well as pathogen virulence. Yet, how this network is influenced and shaped throughout the co-evolution of hosts and their well-adapted pathogens has not been studied in detail.

6 Aims

Many studies have analyzed the co-evolutionary dependency of *NBS-LRR* and corresponding effector genes in oomycete-plant interactions. The evolutionary pattern of one component of this system often matches the evolutionary pattern of the other (Botella et al. 1998, Allen et al. 2004, Rose et al. 2004, Rehmany et al. 2005). However, sRNAs and their regulation add a new level of complexity to this system and the co-evolutionary forces that modulate sRNA-mediated resistance are still unclear. The scope of this thesis is to determine which evolutionary forces act upon this RNA silencing – resistance network. This was done by:

i) analyzing the molecular evolution of the *NBS-LRR* targeting plant miRNA family miR482/2118 and the evolutionary behavior of its targeting network in the Solanaceae to carve out its importance in the evolution of resistance responses.

ii) studying evolutionary patterns and expression of the conserved *Phytophthora* RNA silencing suppressor PSR2 in *P. infestans* to better understand the evolution of RNA silencing suppressors and their role during pathogen infection.

7 Results and Discussion

R and effector proteins have been the focus of numerous studies on plant-pathogen interactions. The role of RNA silencing, however, has, for a long time, only featured in investigations that revolved around plant-virus interactions (cf. Zhao et al. 2016). The finding that bacteria also possess and use RNA silencing suppressors to enhance their virulence (Navarro et al. 2008) has raised the awareness for miRNAs in other areas of phytopathology. Global shifts in miRNA expression have been observed in response to many pathogens since then (e.g. Guo et al. 2011, Pérez-Quintero et al. 2012, Yang et al. 2013). Additionally, several miRNA families were found to target and regulate *NBS-LRR* gene expression (e.g. Li et al. 2012, Shivaprasad et al. 2012, Ma et al. 2014) or derive from *RxLR* and *CRN* genes (Vetukuri et al. 2012, Fahlgren et al. 2013, Åsman et al. 2016).

Of all *NBS-LRR* regulating miRNAs, the miRNA family miR482/2118 has been studied the most, because i) it is distributed throughout many plant families (Zhai et al. 2011, Shivaprasad et al. 2012, Publication I), ii) it is potentially able to regulate many *NBS-LRRs* at the same time (Zhai et al. 2011, Shivaprasad et al. 2012, Publication I), iii) it induces phasiRNA production followed by *cis*- and *trans* regulation of other transcripts, including *NBS-LRRs* (Zhai et al. 2011) and iv) it shows a low conservation of the mature miRNA regions between paralogs, which has not been reported for any other miRNA family (Shivaprasad et al. 2012).

A common observation was that, upon inoculation with a pathogen, down-regulation of miR482/2118 and up-regulation of corresponding *NBS-LRR* targets occurred (Li et al. 2012, Shivaprasad et al. 2012, Boccara et al. 2014, Ouyang et al. 2014). Down-regulation of miR482/2118 was suggested to be a result of the action of RNA silencing suppressors secreted by the pathogen (Shivaprasad et al. 2012). The resulting *NBS-LRR* up-regulation was associated with increased defense responses (Boccara et al. 2014). What this implicates is that through miR482/2118 down-regulation by the pathogen, NBS-LRR protein levels automatically and inevitably increase, resulting in an enhanced defense response (cf. Pumplin and Voinnet 2013). Therefore, miR482/2118 was suggested to have evolved as a counter-counter defense mechanism of plants against global RNA silencing suppressors encoded by many pathogens (Shivaprasad et al. 2012). However, besides a direct role during a plant-pathogen interaction, other hypotheses have been proposed. Fei and colleagues (2013) suggested an additional role in regulating plant-symbiont interactions through up-regulation of the miRNA family, followed by down-regulation of resistance responses. They also suggested a role in expressional buffering. This means the suppression of *R* gene expression via miRNAs in times where there is no pathogen challenge to i) avoid high fitness costs of *R* gene mis-expression (Fei et al. 2013) and ii) retain the ability to rapidly respond to pathogen attack through a release of the miRNA

regulation (Fei et al. 2013). This is supported by studies in *Arabidopsis,* showing that mis-expression of *R* genes indeed has a high cost for plant fitness (Tian et al. 2003, Kim et al. 2010). Additionally, negative regulation of *R* genes may also contribute to *R* gene evolution (Fei et al. 2013): miR482/2118 may buffer spontaneously arising mutations in *NBS-LRRs* to prohibit direct deleterious effects (such as constitutive induction of immune responses). By this, potential evolutionary constraints on *NBS-LRR* genes are lowered, which can also create advantageous *NBS-LRR* alleles (Fei et al. 2013). Additionally, miRNA targeting may promote duplication of *NBS-LRR* genes (Zhang et al. 2014, González et al. 2015), because they suppress the additional *R* transcript abundance resulting from a duplication.

7.1 miR482/2118 – a versatile family of *R* gene-regulating microRNAs

Within the Solanaceae, eight different miR482/2118 family members have been characterized (**Publication I**). Their diversification began early in the Solanaceae lineage (**Publication I**). Moreover several pathogens of cultivated and wild tomatoes encode RNA silencing suppressors (Zrachya et al. 2007, Navarro et al. 2008, Qiao et al. 2013). This makes the miR482/2118 family members of the Solanaceae good candidates to study their evolution alongside their impact on plant pathogen co-evolution. Our evolutionary studies on the miR482/2118 family showed that the eight miR482/2118 family members were subjected to different evolutionary constraints (**Publication I**); often evolutionary rates, especially in the mature miRNA regions, but also in the surrounding regions, were much higher than expected for an ancient miRNA (Fahlgren et al. 2010, **Publication I**), such as miR482/2118 (Xia et al. 2015). This observation is in agreement with the generally high evolutionary rates in *R* genes (Clark et al. 2007). Additionally, the variability in the mutation rates of the mature miRNA region in the miRNA paralogs mirrors the variability in *R* gene paralog evolution (Kuang et al. 2004, **Publication I**). I therefore hypothesize that the evolution of the miR482/2118 orthologs is linked to the evolution of their targets within a species and is, hence, lineage-specific (**Publication I**). Moreover, additional miR482/2118-like sRNAs have been detected in *S. lycopersicum* sRNA transcriptomes (Shivaprasad et al. 2012). These sRNAs could not be mapped to the *S. lycopersicum* genome (**Publication I**). Because some of these variants had a higher read abundance than the original sequence (Shivaprasad et al. 2012), it suggests that the miR482/2118 repertoire shows cryptic variation.

The miR482/2118 family targets 20% of the *NBS-LRR* repertoire in *S. lycopersicum* (**Publication I**). This may not seem significant, given that *S. lycopersicum* has 260 predicted *R* genes (Suresh et al. 2014). However, the phasiRNA network that acts in *cis* and *trans* (Zhai et al. 2011) probably further extends this miRNA-*NBS-LRR* targeting network. In this context, it is striking that the same percentage of *NBS-LRRs* in *S. lycopersicum* and *S. tuberosum* are targeted by miR482/2118

(Publication I), despite *S. tuberosum* having 174 more *NBS-LRR* genes present in its genome (Lozano et al. 2012, Suresh et al. 2014), as well as an additional miR482/2118 member in comparison to *S. lycopersicum* (Publication I). This indicates the necessity of a robust fraction of *NBS-LRR* targets in *Solanum* species to control *NBS-LRR* regulation.

To better understand the robustness of this network, we have analyzed the targeting of the miR482/2118 in an evolutionary context (Publication I). We found a positive correlation between *R* gene family size and numbers of miRNA paralogs targeting a particular *R* gene family (Publication I). This is in agreement with the studies from Zhang et al. 2014 and González et al. 2015, indicating that miRNA targeting promotes *R* gene duplication and hence *R* gene evolution. In addition, we observed a versatility in the miRNA-target relationships: paralogous *NBS-LRRs* are mainly not targeted by the (exact) same set of miR482/2118 members and likewise orthologous *NBS-LRRs* are mainly not targeted by the (exact) same set of miR482/2118 orthologs (Publication I). I hence hypothesize that this ability to dynamically change targeting relationships, without releasing the target's suppression, together with the promotion of duplication, allows for a relaxed evolutionary constraint in miR482/2118 targets (Publication I).

7.2 PSR2 – a master regulator to globally modify host signaling

The presence of a versatile miR482/2118 family enables the plant to rapidly react to the evolution of new pathogen effectors and minimizes the chance of direct negative effects to the plant. At the same time miR482/2118 has been observed to be down-regulated in *S. lycopersicum* upon treatment with several viruses (Shivaprasad et al. 2012), *P. syringae* (Shivaprasad et al. 2012) and the fungus *Fusarium oxysporum* (Ouyang et al. 2014). The corresponding *NBS-LRR* targets were shown to be up-regulated (Ouyang et al. 2014), supporting a direct role for miR482/2118 in infections. It was further shown that down-regulation of miR482/2118 directly suppresses *P. syringae* growth and is both, a PTI and an ETI response (Boccara et al. 2014). This agrees with the concept of Shivaprasad and colleagues (2012) that the miRNA response is a counter-counter defense in the co-evolution of plants and pathogens (Figure 3).

In light of this, we analyzed the evolution of the oomycete RNA silencing suppressor PSR2 in *P. infestans* (Publication II). PSR2 suppresses phasiRNA production (Figure 3a, Qiao et al. 2013). It is one of the few effectors present in all analyzed *Phytophthora* species and the closely-related downy mildew species *H. arabidopsidis* (Xiong et al. 2014). Moreover, PSR2's function as an RNA silencing suppressor is conserved among the pathogen species (Xiong et al. 2014), emphasizing its importance for *Phytophthora* spp. virulence. In agreement with this, *in planta* expression of Ps*PSR2* makes the plant more susceptible to infection by several *Phytophthora* pathogens (Qiao et al. 2013, Xiong et al.

2014). However, the evolutionary rates of PSR2 in the *Phytophthora* species *P. sojae, P. parasitica* and *P. infestans* differ within species, with *P. infestans* showing an intermediate rate (**Publication II**). This difference in the evolutionary rates of PSR2 positively correlates with the broadness of the respective pathogen's host range: *P. sojae* only infects soybean (Kuan and Erwin 1980, Pratt 1981, Crawford et al. 1996), *P. infestans* infects several members of the Solanaceae (Adler et al. 2002, Flier et al. 2003, Garry et al. 2005) and *P. parasitica* has been reported as a pathogen on many, diverse plant genera that belong to 42 different flowering plant families (Hickman 1958). This indicates that not only the miR482/2118 members evolve lineage-specific to sustain *R* gene targeting and promote *R* gene evolution (**Publication I**), but that PSR2 has also evolved lineage-specific to ensure host-specific RNA silencing (**Publication II**).

The finding that overexpression of Ps*PSR2* in *Glycine max, N. benthamiana* and *A. thaliana* increases the success of *Phytophthora* infection (Qiao et al. 2013, Xiong et al. 2014) not only underlines the importance of this effector for *Phytophthora* spp., but also supports the assumption that PSR2 targets a conserved protein or pathway in its various hosts. A target within the host's RNA silencing machinery is, therefore, likely: The core components of the RNA silencing machinery are present throughout the land plants (Zong et al. 2009, Mukherjee et al. 2013, Zhang et al. 2015). Mutations in or silencing of these core components show strong effects on plant fitness (development and defense) up to mortality (Hendelman et al. 2013, Verlaan et al. 2013, reviewed in Bologna and Voinnet 2014, Kravchik et al. 2014). This suggests only limited room for the plant proteins to adapt to a pathogen effector. A target and effector gene often show similar evolutionary histories (Botella et al. 1998, Allen et al. 2004, Rose et al. 2004, Rehmany et al. 2005) and in agreement with this, our population genetic analyses of Pi*PSR2* indicated that the effector gene was under purifying selection with a relaxed constraint (**Publication II**). However, the observed aa polymorphisms had no effect on the protein structure prediction, independent of whether they were considered radical changes or not (**Publication II**). *Pi*PSR2 and its homologs in other *Phytophthora* species were shown to possess several W- and Y-motifs (Ye and Ma, 2016). These motifs are considered to add robustness to an effector protein structure, allowing for adaptive changes to occur without influence on the protein structure (Jiang et al. 2008, Boutemy et al. 2011). We noticed that 40% of the changes mapped to W- and Y motifs in the *Pi*PSR2 sequence (**Publication II**). This indicates that the changes that we observed are not deleterious and may have resulted from slight adaptations to different *Solanum* species or even cultivars; which is in agreement with our observation that host range correlates with PSR2 protein diversity (**Publication II**). Overall our study suggests that *PSR2* is not unable but limited in its evolutionary capacity to rapidly adapt to host molecules such as R proteins, making it a good target to study for *P. infestans* management.

Besides mutation there are other means by which pathogens can modulate their virulence on rapid evolutionary time scales: isolate-specific effector expression patterns were associated with virulence of *P. sojae* and *P. infestans* isolates (Gilroy et al. 2011, Qutob et al. 2013). Hence, to better understand at which time point during infection PSR2 functions and if it can easily be altered in an isolate- or host-dependent manner, we assayed expression of Pi*PSR2* during infections of wild and cultivated tomatoes with a weak and two strong isolates (**Publication II**). In our study, we observed that Pi*PSR2* was expressed from establishment of the biotrophic phase onwards; expression was generally higher in the weak isolate compared to the strong isolates (**Publication II**). Similarly, Wang et al. (2011) observed that overexpression of a biotrophic *RxLR* effector led to decreased virulence, suggesting that correct Pi*PSR2* expression during the biotrophic phase is important for virulence of *P. infestans* isolates. Over time Pi*PSR2* was either constantly expressed or down-regulated, especially during the transition phase from biotrophy to necrotrophy depending on the isolate and host (**Publication II**). Additionally, we found a negative correlation between the expression of a *P. infestans* biomass marker and the expression of Pi*PSR2* in two out of three isolates (**Publication II**). This suggests that i) the Pi*PSR2* expression level determines virulence and ii) the expression pattern depends on pathogen isolate and host. Results obtained by Xiong and colleagues (2014) further support this observation. Their study demonstrated that in *P. sojae* Ps*PSR2* is up-regulated during the transition phase from biotrophy to necrotrophy, rather than vice versa as we have seen for *P. infestans;* hence *PSR2* expression is not only isolate dependent, but also differs between *Phytophthora* species (**Publication II**).

But what does *PSR2* expression tell us about its influence of phasiRNA production? PSR2 does not globally regulate phasiRNAs (Qiao et al. 2013), but only those that are initiated by a binding of a 22 nt miRNA to the target transcript (one-hit model; Chen et al. 2010, Cuperus et al. 2010, Fei et al. 2013, Qiao et al. 2013). The *NBS-LRR*-derived phasiRNAs are also generated by this mechanism (Zhai et al. 2011). Hence up-regulation of the RNA silencing suppressor *PSR2* during the biotrophic phase of *P. infestans* suggests a reduction in phasiRNA levels, including those derived from *NBS-LRRs* (Figure 3b). Before, I argued that *NBS-LRR*-derived phasiRNAs extend the network of *R* gene targeting established by miR482/2118. Therefore, a plausible conclusion is that a reduction in these phasiRNAs leads to an increase of *NBS-LRR* transcripts and ultimately to an enhanced defense response in the early infection phase (Figure 3b). However, the phasiRNA pathway is a critical regulator of many plant signaling pathways, including development, abiotic stress and nutrient signaling (Hsieh et al. 2009, Zhai et al. 2015, Zheng et al. 2015). Factors such as increased nutrient uptake or slightly altered developmental settings that may locally modify source-sink relationships are profitable for the pathogen in its biotrophic phase. Additionally, phasiRNA regulation can be plant-, and therefore host-

specific (Zheng et al. 2015). I, therefore, conclude that due to PSR2's i) ubiquitous presence, ii) within-species evolutionary rate and iii) its early on-set of expression, this effector is a master regulator designed to allow all *Phytophthora* species to modify similar plant signaling pathways in different hosts (Publication II). By that the positive effects, such as altered nutrient signaling, outweigh the negative effects, such as an early increased resistance response.

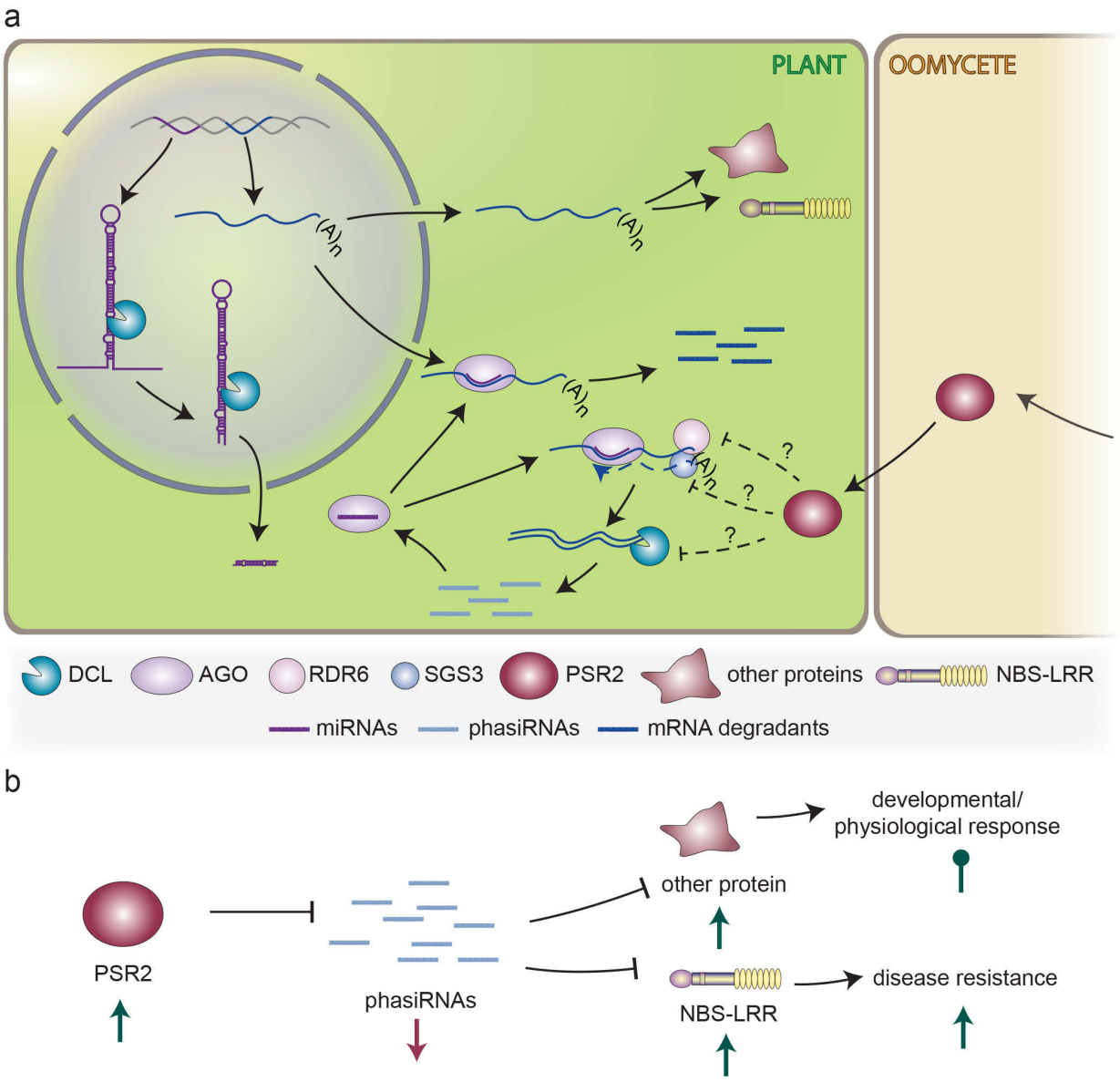

Figure 3. The RNA silencing network during plant-oomycete interactions. (a) In the plant, pri-miRNAs are transcribed and then processed by DCL1 into pre-miRNAs and then into mature miRNAs. The mature miRNAs are incorporated into AGO proteins and targeted to mRNAs. This miRNA-targeting leads to either mRNA degradation or recruiting of the RDR6/SUPPRESSOR OF GENE SILENCING 3 (SGS3) complex. The RDR6/SGS3 complex generates dsRNA, which is further processed by DCL4 into phasiRNAs. phasiRNAs are incorporated into AGO proteins and target other (m)RNAs in *cis* and *trans.* Oomycete pathogens can secrete PSR2 proteins into the plant cytoplasm, where they interfere with the phasiRNA biosynthesis. Dashed lines with question marks point towards plant proteins that are potentially suppressed by PSR2. This suppression by PSR2 is predicted to enhance the translation of phasiRNA-regulated mRNAs into NBS-LRRs and other proteins. This will lead to developmental and physiological responses but also enhanced immunity. (b) We observed that during the early infection stages an up-regulation of *P. infestans PSR2* transcripts (conceivably resulting in more PSR2 protein) occurred. Elevation of PSR2 protein levels likely lead to decreased levels of phasiRNAs. Under this scenario, suppression of translation of NBS-LRRs and other proteins is lifted, increasing resistance and altering developmental as well as physiological responses.

8 Conclusion

P. infestans is a major pathogen of Solanaceae crops and their wild relatives. To understand the role of RNA silencing in the *Phytophthora-Solanum* co-evolution we analyzed i) the evolutionary patterns and roles of the *R* gene targeting miRNA family miR482/2118 and ii) the newly identified oomycete RNA silencing suppressor PSR2. Both components show lineage-specific evolutionary rates (**Publication I**, **Publication II**). Yet, while the versatility of miR482/2118 promotes *R* gene evolution to counter-act pathogen effectors, PSR2 appears as an invariable constant in plant-oomycete interactions (**Publication I**, **Publication II**). The evolutionary analyses on miR482/2118 and its targeting suggest that miR482/2118 acts as an evolutionary buffer that on one hand promotes *R* gene evolution and on the other hand limits negative fitness effects for the plant through a constant suppression of newly arising variants (**Publication I**). PSR2's conserved presence, function (Xiong et al. 2014) and its expression pattern during infection (**Publication II**) highly favors the counter-counter defense hypothesis for the role of miR482/2118 and their subsequent phasiRNAs. To summarize this, the results of **Publication I** and **Publication II** support both hypotheses on the role and function of sRNA regulation of *R* genes in long-term plant-pathogen interactions. As in many other plant-pathogen interactions, the RNA silencing network is an integral and decisive component of the evolution of the *Phytophthora-Solanum* pathosystem.

9 Summary of the obtained results

9.1 Publication I: Evolutionarily dynamic, but robust, targeting of resistance genes by the miR482/2118 gene family in the Solanaceae.

Diverse microbes can be found both on and in plants. Yet, only few of them cause plant diseases. For a plant, constant presence of R proteins might therefore be more costly than profitable (Tian et al. 2003, Kim et al. 2010). Nevertheless, in the case of infection, R protein levels should be up-regulated as quickly as possible. One way to achieve this is through the negative regulation of *R* gene expression by miRNAs. In this publication, we analyzed the distribution, evolution and role of the miR482/2118 family in 14 Solanaceae species. Eight different miR482/2118 types have been found in total. Seven of these were conserved within the wild tomatoes. Each type has its own, unique evolutionary pattern, suggesting a lineage-specific evolution. The subset of *NBS-LRR* genes targeted by miR482/2118 is constant in the two *Solanum* species *S. lycopersicum* and *S. tuberosum*. In contrast, the specific targeting of the miR482/2118 members displayed high versatility. This adds a certain dynamic and species-specific component to the *NBS-LRR* regulatory network. Additionally, we found that large *NBS-LRR* families are associated with more miR482/2118 members targeting them. Taken together the data indicate that miR482/2118 promotes *NBS-LRR* evolution because it acts as an evolutionary buffer for *R* genes.

9.2 Publication II: Signatures of selection and host adapted gene expression of the *Phytophthora infestans* RNA-silencing suppressor PSR2

P. infestans is an oomycete pathogen of several Solanaceae species. Its close relatives of the *Phytophthora* genus are likewise detrimental for their hosts. Recently an RxLR effector of these species was identified as an RNA silencing suppressor (PSR2) of their host plant's RNA silencing machinery (Qiao et al. 2013). PSR2 is special as it is one of the few effectors that has orthologs in many *Phytophthora* species and even in the downy mildew *H. arabidopsidis* (Win et al. 2007, Haas et al. 2009, Xiong et al. 2014). In this study we set out to better understand how RNA silencing suppressors evolve in the co-evolutionary arms race between plants and pathogens by studying i) the evolutionary history of PSR2 and ii) its expression during *P. infestans-Solanum* interactions. We found a lineage-specific evolution of PSR2 in the different *Phytophthora* species that correlated with the pathogens host ranges. Comparing 16 different *P. infestans* isolates we determined that Pi*PSR2* evolved under purifying selection with a relaxed evolutionary constraint. This is in agreement with my assumption that PSR2 targets an integral component of the RNA silencing machinery. Expression analyses further suggest that elevated Pi*PSR2* expression reduces virulence. All expression patterns of Pi*PSR2,* despite being isolate- and host-specific, indicate a role for PSR2 in the early infection phase. This points to PSR2 being a master regulator of phasiRNAs, supporting the counter-counter defense hypothesis put forward by Shivaprasad et al. (2012) concerning miRNAs and phasiRNAs that regulate *NBS-LRRs.*

10 Publications associated with this thesis

10.1 Publication I: Evolutionarily dynamic, but robust, targeting of resistance genes by the miR482/2118 gene family in the Solanaceae.

Authors: Sophie de Vries, Thorsten Kloesges and Laura E. Rose

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Sophie de Vries' contribution:

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Evolutionarily Dynamic, but Robust, Targeting of Resistance Genes by the miR482/2118 Gene Family in the Solanaceae

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Abstract

Plants are exposed to pathogens around the clock. A common resistance response in plants upon pathogen detection is localized cell death. Given the irreversible nature of this response, multiple layers of negative regulation are present to prevent the untimely or misexpression of resistance genes. One layer of negative regulation is provided by a recently discovered microRNA (miRNA) gene family, miR482/2118. This family targets the transcripts of resistance genes in plants. We investigated the evolutionary history and specificity of this miRNA gene family within the Solanaceae. This plant family includes many important crop species, providing a set of well-defined resistance gene repertoires. Across 14 species from the Solanaceae, we identified eight distinct miR482/2118 gene family members. Our studies show conservation of miRNA type and number in the group of wild tomatoes and, to a lesser extent, throughout the Solanaceae. The eight orthologous miRNA gene clusters evolved under different evolutionary constraints, allowing for individual subfunctionalization of the miRNAs. Despite differences in the predicted targeting behavior of each miRNA, the miRNA–*R*-gene network is robust due to its high degree of interconnectivity and redundant targeting. Our data suggest that the miR482/2118 gene family acts as an evolutionary buffer for *R*-gene sequence diversity.

Key words: microRNA, gene family evolution, disease resistance, *R*-genes, miR482/2118, wild tomatoes.

Introduction

Pathogens can exert strong natural selection on their hosts. Since potential pathogenic organisms are widespread in plant communities, plants require a fine-tuned and variable defense system. Essential components of the plant immune system are receptor proteins coupled with signaling proteins that detect pathogen molecules and subsequently mount a defense response. Of course, counter-adaptation by the pathogen leads to selection for pathogen expressed molecules that interfere with host detection or downregulate immune system genes.

A major class of R (resistance) proteins is represented by the nucleotide binding site-leucine rich repeats (NBS-LRRs; Meyers et al. 2005). These proteins recognize effectors in a direct or indirect fashion and are able to redirect the defense signaling and elicit *R*-gene-mediated immunity. In turn, NBS-LRR signaling can be undermined by pathogen effectors. Thus, the combination of a variable effector and *R*-gene complement is assumed to underlie the variety in host-pathogen specific interactions (Jones and Dangl 2006).

However, the presence or absence of these factors may not be the only determinant of specificity and orchestration of a timely defense response. Variation at the level of *R*-gene regulation may also contribute to the outcome of a host-parasite encounter. Proper regulation of *R*-genes can be important for the following reasons: 1) misregulation of *R*-genes can result in autoimmune responses and fitness costs in plants (Kim et al. 2010), 2) higher transcript abundance induced upon pathogen attack can lead to improved resistance (Cao et al. 2007; Bradeen et al. 2009; Kramer et al. 2009), and 3) faster transcriptional responses provide advantages in defense responses (Milling et al. 2011). Thus, exquisite and precise control of *R*-gene expression is no less important to the host or to the pathogen, although for opposing reasons.

Recently several microRNAs (miRNAs), 22 nt short small RNAs, have been predicted to target NBS-LRR encoding transcripts (e.g., Zhai et al. 2011; Li et al. 2012; Shivaprasad et al. 2012; Ma et al. 2014). miRNAs are encoded by so-called *MIR* genes. They are transcribed as primary miRNAs (pri-miRNA), which are further processed by a Dicer-like protein into a precursor miRNA (pre-miRNA). These pre-miRNAs are processed into the mature miRNA (Rogers and Chen 2013; Bologna and Voinnet 2014). In total, five different *MIR* gene families have been described to target domains in *NBS-LRR*s genes. One family, miR482/2118, stands out by having high sequence diversity among its family members (Shivaprasad et al. 2012). It consists of the two subfamilies, miR482 and miR2118, which both target the P-loop sequence motif in the *NBS-LRR* mRNA, and can be distinguished in the 5′-end of the mature miRNAs (Shivaprasad et al. 2012). The targeting of *NBS-LRR* mRNA leads to degradation of the *R*-gene transcript and production of phased secondary small interfering RNAs (phasiRNAs). The production of phasiRNAs strengthens the regulatory network by targeting the original and other *NBS-LRRs* for posttranscriptional regulation (Zhai et al. 2011; Shivaprasad et al. 2012).

Previous comparative small RNA transcriptome studies have described rapid evolution of miRNA families, including high birth and death rates via gene duplication, followed by functional redundancy, subfunctionalization, neofunctionalizaton, and pseudogenization (Maher et al. 2006; Nozawa et al. 2012). From genome-wide comparisons of *MIR* genes across several plant species, it has been found that the miRNA region and its complementary region (miR* region) show strong evolutionary constraints, while the surrounding pre-miRNA regions evolve more rapidly, with rates typical of intergenic regions (Fahlgren et al. 2010; Nozawa et al. 2012; Zhao et al. 2015). Most miRNAs target either a specific mRNA or small gene families. The evolution of miRNAs that target a large gene family, such as the *NBS-LRR* gene family, would potentially be exposed to different evolutionary constraints, than miRNAs with a more limited target repertoire.

The regulation of the miR482/2118 genes may be equally important as the regulation of *NBS-LRRs* themselves. Both transcriptional regulation of *MIR* genes and processing of pri-miRNAs to miRNAs can influence miRNA abundance. In plants and animals, nucleotide variation in the loop regions, but also in the 5′, 3′, and the miR/miR* complex, can influence these processes, leading to alternative miRNA sequences or differential miRNA expression (Krol et al. 2004; Liu et al. 2008; Todesco et al. 2012; Wang et al. 2013; Zhu et al. 2013).

In this study, we investigated the evolutionary history of the miR482/2118–*NBS-LRR* regulatory network in Solanaceae. We generated a data set of miR482/2118 genes from 14 species of Solanaceae and identified eight distinct gene types of this family. miRNA type and number are well-conserved in the group of wild tomatoes and, to a lesser extent, across the genus *Solanum*. However, all miRNA genes are predicted to be derived from duplication events predating the *Solanum–Nicotiana* split (~24 Ma), with some even older than the Solanaceae itself, indicating subsequent remodeling of the miRNA repertoire after speciation. Comparisons of estimates of evolutionary rates among miRNA types show type-specific differences in evolutionary constraints: Some genes show the greatest sequence conservation in the miR and miR* regions, while others show the greatest sequence conservation in the 5′- and 3′-precursor regions. Target prediction analyses indicate a robust targeting network within and between species, due to a high target overlap between miR482/2118 genes. However, differential expression of miRNA in planta reveal that despite an overlap in potential targets, subfunctionalization may have played a role during the evolution of this gene family. Evolutionary robustness of this network (through redundancy of targeting) may help to support the rapid evolution of *NBS-LRRs* within tomato and potato.

Materials and Methods

Assembly of miR482/2118 Family Members from the Plant Kingdom

Sequences of the miR482/2118 genes annotated in miRBase v. 20 were downloaded (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008; Kozomara and Griffiths-Jones 2011). This included sequences from 29 plant species. The number of miR482/2118 family members from *Picea abies* was updated from four, reported in miRBase, to 24 recently reported by Xia et al. (2015). Additionally, we searched for these genes from the genomes of *Lotus japonicus* (Sato et al. 2008) and *Brachypodium distachyon* (International Brachypodium Initiative 2010) using nucleotide blast (BLASTn), because information in miRBase on these two species was sparse. All precursors and mature miRNA sequences of miR482/2118 from the Fabaceae and Poaceae available in miRBase v.20 were used as input for the searches. The draft genome of *Mimulus guttatus* (v. 1.0; Hellsten et al. 2013) was queried with the known precursor and mature miRNA sequences from *Solanum lycopersicum* published in Shivaprasad et al. (2012) and miRBase v. 20 to increase the representation of the Asterids in the analyses. To compare the gene family representation across the different species and to evaluate species-specific differences, we recorded the miR482/2118 gene numbers per species on the phylogeny. The phylogeny was based upon the following publications: Catalán et al. 1997; Yang et al. 1999; Wang et al. 2000; Wikström et al. 2001; Wojciechowski 2003; Swigoňová et al. 2004; Lavin et al. 2005; Delgado-Salinas et al. 2006; Van et al. 2008; International Brachypodium Initiative 2010; Pires and Dolan 2012.

In Silico Identification of Additional miR482/2118 Precursor Sequences from the Solanaceae

To ensure that we had not overlooked or excluded any potential gene family members from our analyses, we searched the *S. lycopersicum* genome SL2.40 for matches to miR482/2118 sequences from *S. lycopersicum* and *Solanum tuberosum* identified from miRBase v. 20 and described in Shivaprasad et al. (2012; supplementary table S1, Supplementary Material online). This search was implemented using the PatMan-based sequence alignment tool implemented in the UEA small RNA workbench v 2.5.0 (Prüfer et al. 2008). Up to three mismatches were allowed per 22 nt-hit. We downloaded 450 bp upstream and 450 bp downstream of the predicted miRNA and performed structure predictions using the secondary structure tool of the CLC Main Workbench v. 6, sampling ten suboptimal structures each time. We subsequently shortened the approximately 900 bp to determine a good secondary structure of an appropriate length and then compared our results with the known pre-miR482/2118 sequences for *S. lycopersicum* and *S. tuberosum* from miRBase (supplementary fig. S4, Supplementary Material online). As a final step, we did a BLASTn querying all known/identified precursor miR482/2118 sequences of *S. lycopersicum* in the *S. tuberosum* genome and vice versa using Phytozome v. 9.1 (Goodstein et al. 2012) to ensure that we found the full set of miR482/2118 loci in both sequenced genomes.

To further extend the set of potential precursor sequences from the Solanaceae, we downloaded all miR482/2118 mature and precursor sequences from *Nicotiana tabacum* from miRBase v. 20. Using these sequences, we searched the following nucleotide and expressed sequence tag (EST) databases: *Nicotiana sylvestris* genome (Sierro et al. 2013), *Nicotiana benthamiana* leaf transcriptome (Bombarely et al. 2012), *Physalis peruviana* leaf transcriptome (Garzón-Martínez et al. 2012), *Capsicum annum* genome (Kim et al. 2014), *Solanum melongena* NCBI nucleotide databases, and the draft genome of *Solanum pimpinellifolium* (http://solgenomics.net/organism/Solanum_pimpinellifolium/genome [last accessed July 31, 2014] Ware et al. 2014; supplementary table S1, Supplementary Material online). Structure predictions were done as described above.

Isolation of *MIR* Genes from *Solanum* Species

To increase the number of sequences from the Solanaceae, DNA was extracted from six species of the genus *Solanum*: *Solanum peruvianum* (LA1951 and LA2964), *Solanum chilense* (LA3114), *Solanum corneliomulleri* (LA1274), *Solanum lycopersicoides* (LA2951), *Solanum ochranthum* (LA2682), and *S. melongena* (cv. Black Beauty). The plants were grown under standard greenhouse conditions prior to sampling. DNA extraction was performed as described in Edwards et al. (1991). Precursor sequences were amplified using polymerase chain reaction (PCR). PCR primers were designed to match regions surrounding the precursor sequences and showing high sequence conservation across *S. lycopersicum, S. pimpinellifolium*, and *S. tuberosum*. Twenty microliters PCR reactions were conducted using 1:10 diluted *Solanum* DNA (50–100 ng), 1 × High Fidelity PCR Buffer, 2 mM $MgSO_4$, 0.1 mM dNTPs, 0.2 μM each primer, and 0.05 U/μl Platinum *Taq* High Fidelity (Life Technologies). Primer sequences and annealing temperatures can be found in supplementary table S2, Supplementary Material online. Cycle conditions were according to the manufacturer's instructions with 4 min initial denaturation phase and 7 min final extension. PCR products were purified using peqGOLD Cycle-Pure Kit (peqlab, VWR) according to the manufacturer's instructions. PCR products were cloned using the TOPO TA Cloning Kit for subcloning with electro-competent One Shot TOP10 *Escherichia coli* cells (Life Technologies). Sequencing was performed by Eurofins MWG Operon (Germany). Sequences were aligned to the in vitro identified precursor sequences in MEGA 5.2.2 (Tamura et al. 2011) and quality was checked based on the sequence chromatograms. Secondary structure predictions were done as described above.

Origin of the miR482/2118 Gene Family

MIR genes are proposed to originate along three different routes: from 1) protein-coding genes, 2) transposable elements (TE), or 3) *MIR* gene duplication. To determine which origin accounted for the diversification of the miR482/2118 family in the Solanaceae, we evaluated these three scenarios in turn. To find potential protein-coding sequences that may have resulted in the origin of the ancestral miR482/2118 genes, we conducted a BLASTn using all the genes in figure 1*b* against the NCBI EST, nucleotide and RefSeq databases. For evaluating the possibility of a TE derived ancestor, we used RepeatMasker Open-4.0 with the default settings for the internal libraries for Solanaceae (http://www.repeatmasker.org [last accessed August 4, 2014] Smit et al. 2014). To evaluate a large-scale duplication hypothesis for *MIR* genes, we concentrated on the *MIR* genes found in *S. lycopersicum* and followed the procedure described by Maher et al. (2006): we used a custom designed Perl script to extract the ten closest protein-coding genes on either side of each miR482/2118 precursor location from the *S. lycopersicum* genome. To identify potential paralogs we conducted a BLASTp with the ITAG2.4_proteins_full_desc.fasta from the *S. lycopersicum* SL2.50 release as our database and the extracted protein sequences as our query. Only matches of the query with proteins on chromosome 3, 4, and 6 were included in the further analysis. An e-value cutoff of less than 10e-04 was applied. We then mapped query versus database matches in order of their chromosome location to identify stretches of three or more conserved flanking genes, which would indicate large-scale duplication events. Tandem duplications are indicated by

MIR genes that have the identical ten flanking protein-coding genes on both sides (Maher et al. 2006), that is, no protein-coding genes occur between the two *MIR* genes.

Phylogenetic Analysis

We constructed a phylogeny to display the *MIR* gene duplication history using PHASE 3.0 (Jow et al. 2002; Allen and Whelan 2014). PHASE 3.0 is a phylogenetic program designed to study RNA evolution and was developed for RNA sequences that typically form secondary structures. Precursor sequences were aligned using ClustalW in MEGA 5.2.2 with the default settings (supplementary fig. S2, Supplementary Material online). The precursor sequences were approximately 130 bp long (range 84–171 bp). miR5300 sequences were excluded from the analysis because of their substantially longer precursor sequences (~265 bp) and their partial switch of the 5′- and 3′-precursor arm. The gene Ghr-miR482a from cotton was chosen as an outgroup because its mature sequence (22 nt) is identical to sly-miR482f and it shares substantial similarity across the entire precursor with sly-miR482f (Shivaprasad et al. 2012). We performed model selection using a PHASE 3.0 implemented Perl script. The necessary secondary consensus structure of the precursor data set was created using a web-based version of RNAalifold (Bernhart et al. 2008; Gruber et al. 2008).

To infer the phylogeny, we applied a Markov–Chain–Monte–Carlo (MCMC) model based on two substitution models implemented in PHASE 3.0. Model 1 (REV + G with five gamma categories) is applied to unpaired bases of the predicted RNA structure. This substitution model is similar to those used for DNA sequences. Model 2 (RNA16D + G with five gamma categories) is applied to predicted paired regions in the secondary structure. Using two different substitution models for paired and unpaired regions, we can take into account the influence of secondary structure on the evolution of the molecule. We used 750,000 burn-in iterations and 1,500,000 sampling iterations with a sampling period of 150. We initiated the analysis with 11 different random seeds and afterwards computed the corresponding consensus trees using the mcmcsummarize function. All consensus trees resulted in the same clades with similar Bayesian probability support values. A subsequent analysis was conducted to investigate the paraphyletic relationship of the miR482 clade. A maximum likelihood tree based exclusively on the underlying nucleotide alignment and ignoring the secondary structure information was inferred assuming the K2 + G substitution model with five gamma categories.

Estimating the Dates of miR482/2118 Duplication Events

To estimate the minimum age of the duplication events, we used MrBayes v 3.2.2 to determine whether the genes behaved according to a molecular clock (Ronquist et al. 2012). We assumed the 4by4 nucleotide model, mixed

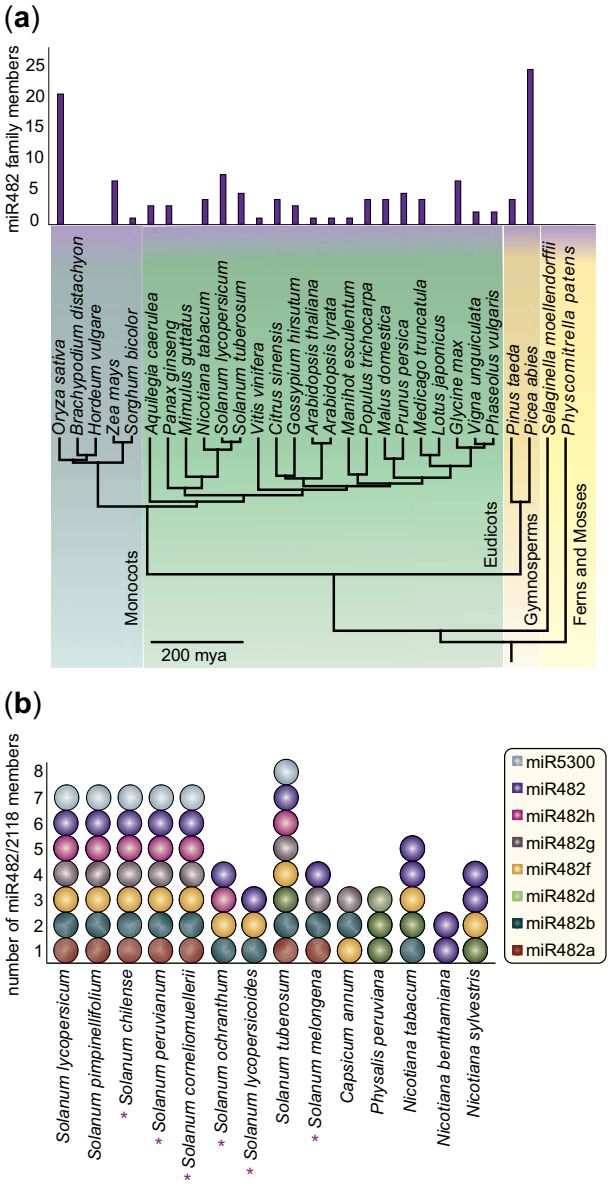

Fig. 1.—The repertoire of miR482/2118 in the plant kingdom. (*a*) Depicted are the number of miR482/2118 sequences for 29 plant species (based on literature, miRBase v. 20 and publically available genomes of *Mimulus guttatus, Lotus japonicas,* and *Brachypodium distachyon*) whose relationships are displayed in a literature-based phylogeny including all major land-plant lineages. (*b*) Presence and distribution of the miR482/2118 gene family across the Solanaceae. Purple asterisks indicate species in which some or all of the sequences were generated in this study.

number of substitution types and gamma-distributed substitution rates with five categories. The strict molecular clock test was performed as described in the manual, however the number of generations was increased to 2,000,000. A molecular clock was rejected for the full data set; however, the removal of the miR482h clade, two *C. annum* sequences (Can-miR482b and Can-miR482g) and one *P. peruviana*

sequence (Ppe-miR482c) resulted in a data set in which a strict molecular clock was the preferred model. Therefore, we inferred a phylogeny for this reduced data set. We estimated a rooted MCMC tree assuming a molecular clock in PHASE 3.0 based upon the previously described settings for the unrooted MCMC tree. We used the consensus tree output from the reduced data set as an input and allowed for no alterations in the phylogeny.

Nucleotide Divergence in the miR482/2118 Family Members

We determined the average nucleotide divergence per site/per million years for the individual members in the miR482/2118 family in the Solanaceae. The sequences were first divided into precursor groups based on their clustering in the phylogeny. Then five precursor regions were determined within each precursor group: 5′-stem region, miR* region, loop region (between miR and miR*), miR region, and 3′-stem region. We determined the miR* region for each precursor sequence separately based on the precursor's secondary structure. Each precursor group was aligned in ClustalW in MEGA 5.2.2 with the same settings as described above. Pairwise divergence between the precursor sequences within a precursor group was calculated. The number of fixed differences was estimated as described in Fahlgren et al. (2010). The number of fixed differences was divided by the length of the longer sequence in each pair. This was then divided by twice the divergence time in Myr, to calculate the substitution rate. These rates were averaged within a precursor group. For comparison, we did the same analysis on the miR166 family in the Solanaceae, collecting the known sequences deposited in miRBase.

Target Prediction

To determine the potential targets of miR482/2118 miRNA genes in *S. lycopersicum* and *S. tuberosum,* we used the software psRNATarget: a plant small RNA target analysis server (Dai and Zhao 2011). As miRNA library input, all miR482/2118 from *S. lycopersicum* and *S. tuberosum* were used. As target library input, the coding sequences from *R*-genes annotated in *S. lycopersicum* and *S. tuberosum* were used (Lozano et al. 2012; Suresh et al. 2014). We conducted the analyses under two settings: a strict setting where the maximum expectation was set to $\leq$2.0 (i.e., low probability of false positives) and a less stringent setting where sequences with a score $>$2.0–3.0 were allowed.

Target Homology and Network Visualization

The homology of the potential targets of miR482/2118 in *S. lycopersicum* and *S. tuberosum* to one another within and between species was determined using orthoMCL (Li et al. 2003; Fischer et al. 2011). The miRNA targeting and the evolutionary relationships of these putative targets were visualized in a network. The network was generated with Cytoscape 3.1.1 (Shannon et al. 2003). Evolutionary relationships between orthologous and paralogous target genes are displayed as undirected edges (i.e., lines). The predicted target-miRNA relationships are displayed as directed edges (i.e., arrows). Potential targets were grouped into gene families according to the predictions of orthoMCL. We determined gains and losses of potential miRNA targets in an evolutionary context. The network was clustered to see whether the miRNAs or the gene families are the major hubs of the network using the Fuzzifier clustering option in the clusterMaker package to allow nodes to belong to more than one cluster (Morris et al. 2011).

Tissue-Specific Expression Patterns in *S. lycopersicum* and *S. pimpinellifolium*

We sampled roots and leaves of *S. lycopersicum* cv. M82 and *S. pimpinellifolium* LA114 to determine the expression of the mature miR482/2118 members in these plant tissues. Detection of the mature miRNA using quantitative PCR indicates that the miRNAs are present and correctly excised in the sample. These species can be grown axenically in the lab and both species are selfing, thereby avoiding issues caused by allele-specific differences in expression. The seeds were surface sterilized by treating them with 70% ethanol for 3 s, followed by 30 s approximately 5% sodium hypochlorite solution and then washed three times in sterilized water. Seeds were transferred on 1% H_2O-agar, incubated in the dark at 18 °C to break the dormancy. Ten days postgermination (dpg) seedlings were transferred to 0.5% MS medium (Murashige and Skoog 1962), containing 1% sucrose. Samples were taken from 23 dpg old plants. We sampled four leaves from four different plants per biological replicate. The entire root (cut at the base of the hypocotyl) was sampled from four plants per biological replicate. Three biological replicates were conducted for the leaf sample and two biological replicates were conducted for the root sample.

RNA was extracted using the Gene Matrix Universal RNA/miRNA purification kit (Roboklon). cDNA was synthesized with the miScript II RT Kit (Qiagen) using the HiFlex buffer. The samples were diluted 1:100 and qPCR was performed with the miScript SYBR Green PCR Kit (Qiagen) in a 25 µl reaction in a CFX Connect Real-Time System (Bio Rad). Primers were designed based on the mature miR482/2118 sequences (supplementary table S2, Supplementary Material online). Three reference genes (TIP41, SAND, and AP2-complex subunit mu-1) were chosen based on Expósito-Rodríguez et al. (2008) and Dekkers et al. (2012). The reference gene primers were designed manually (for gene accessions and primer sequences see supplementary table S2, Supplementary Material online). The reaction protocol was used as specified for the miScript SYBR Green PCR Kit (Qiagen), with exception of the miR482a primer for which the annealing temperature

was increased to 65 °C. The results were evaluated using the method described by Pfaffl (2001).

We tested whether the relative gene expression for each mature miRNA across the replicates was normally distributed. Differences in expression between mature miRNAs in the leaves versus roots of *S. pimpinellifolium* were analyzed using a two-tailed t-test with the assumption of unequal variances. A Mann–Whitney *U* test (Mann and Whitney 1947) was performed to determine whether mature miRNA transcript levels differed significantly between *S. lycopersicum* cv. M82 and *S. pimpinellifolium*.

Results

Distribution of miR482/2118 in Land Plants

To investigate the evolutionary history of the miR482/2118 family, we first determined the distribution of these genes in a subset of plant species based on publicly available data (see Materials and Methods, fig. 1*a*). The miR482/2118 family appears to be absent in mosses and Lycopods (*Physcomitrella patens* and *Sellaginella moellendorfii)* emerging first in the Gymnosperms, followed by an extensive radiation (fig. 1*a*). The gene family is also missing in some seed plants, although their close relatives possess several members (e.g., *M. guttatus* and *L. japonicus*; fig. 1*a*). In the species in which the gene family is present, it is typically represented by one to five members. The species *S. lycopersicum, Glycine max,* and *Zea mays* lie slightly above this with seven to eight family members. Certain species have many more members, such as *Pi. abies* with 24 and *Oryza sativa* with 21 family members (fig. 1*a*).

Distribution of miR482/2118 in the Solanaceae

To gain insight into the more recent evolutionary history of the miR482/2118 family, we focused on plant species in the Solanaceae. To ensure that we had a complete repertoire of the miR482/2118 family in *S. lycopersicum* and *S. tuberosum,* we retrieved eight previously published mature miRNA sequences from *S. lycopersicum* and two miRNAs unique to *S. tuberosum* (Shivaprasad et al. 2012; miRBase v. 20). Mapping these small reads to the *S. lycopersicum* reference genome resulted in 33 intergenic and 61 protein-coding sequences, which may be putative miRNA targets. Of the protein-coding sequences, 48 encode proteins having an NBS-LRR domain. Because none of the protein-coding sequences resulted in promising RNA secondary structures, they were not considered to be putative miRNA genes.

Of the 33 intergenic sequences, seven resulted in promising RNA secondary structures, one of which had not been previously identified in either of the two species, hereafter known as miR482h (supplementary fig. S1, Supplementary Material online). The sly-miR482c, sly-miR482d, sly-miR482e, and sly-miR482f genes mapped to the same region of the *S. lycopersicum* genome (Chr. 4, on position 55142317–55142338), with sly-miR482f showing no mismatches.

The BLASTn query of the precursor sequences yielded three additional genes (miR482f, miR5300, and miR482h) in *S. tuberosum,* but no genes in addition to those known from *S. lycopersicum.* No homologous sequences for stu-miR482b could be found in *S. lycopersicum*, not in the small read alignment, nor in the BLASTn query with the precursor sequences. Homologous sequences for sly-miR482, sly-miR482a, sly-miR482b, sly-miR482f, sly-miR482g, sly-miR482h, and sly-miR5300 could be found in *S. pimpinellifolium*, but not for stu-miR482b (fig. 1*b*). Analyses of members of the *Nicotiana* genus revealed five miR482/2118 members in *N. tabacum,* four members in *N. sylvestris,* and two members in *N. benthamiana* (fig. 1*b*). EST, genome and transcriptome analyses resulted in one additional precursor sequences for *S. melongena,* three for *Capsicum annum* and three for *P. peruviana* (fig. 1*b*). Our PCR approach identified seven miR482/2118 family members for *S. peruvianum, S. chilense, S. corneliomulleri,* four members in *S. ochranthum,* three members in *S. lycopersicoides*, and three members for *S. melongena*. The total miR482/2118 data set generated from the Solanaceae is comprised of 71 precursor sequences.

The miR482/2118 Gene Family Diverged Prior to Speciation within the Genus *Solanum*

We reconstructed the evolutionary history of these genes from the Solanaceae using a Bayesian method assuming evolutionary models including both nucleotide substitution and RNA secondary structure. The individual genes form well-supported monophyletic clades, with the exception of one, miR482 (fig. 2). Because each clade includes genes from multiple species of Solanaceae, it is likely that the gene family diversified well before the divergence of these species.

The absence of reciprocal monophyly for miR482 and miR482h was further investigated. Reciprocal monophyly of these two genes is supported in an analysis based on sequence evolution, but excluding secondary structure (supplementary fig. S3*a*, Supplementary Material online). The secondary structure prediction of the miR482 gene from tomato and the miR482h gene from potato share high similarity, despite many differences in their underlying nucleotide sequences (supplementary fig. S3*b*, Supplementary Material online). We hypothesize that convergent evolution (or homoplasy) in this character confounds phylogenetic inference. The presence of these genes in tandem arrangement in syntenic positions in both plant genomes further supports the scenario that these two genes were present in the ancestor of tomato and potato (supplementary fig. S4, Supplementary Material online). Therefore, in view of the phylogenetic evidence and positional information, we can confidently assert that the miR482/2118 gene family diversified prior to speciation within *Solanum.*

The chronogram also indicates that the miR482/2118 gene family expanded prior to speciation of the major crown lineages in the Solanaceae (fig. 3). The diversification of this family likely occurred more than 24 Ma (the age of the *Nicotiana–Solanum* split, Wu and Tanksley 2010; Särkinen et al. 2013). Only miR482h is found exclusively in tomatoes and potatoes, thus appearing to be of more recent evolutionary origin. In contrast, miR482d is found across several representatives of the Solanaceae, but is missing from the wild tomato clade (figs. 1*b* and 2). These patterns indicate that gene retention and, to some extent, gene loss has occurred within the Solanaceae.

We considered the three postulated origins for *MIR* genes: from 1) protein-coding genes, 2) TE, or 3) *MIR* gene duplication. No evidence for protein origin or TE transposition was found for the miRNA genes in *Solanum.* Maher et al. (2006) estimated the upper limit to detect historical duplication to be approximately 39 Ma. If these miRNA genes duplicated and diverged prior to the split of *Nicotiana* and *Solanum* (estimated to be ~24 Ma), we may have some power to detect these ancestral duplications. However, because no trace in the regions surrounding the miR482/2118 loci in the *S. lycopersicum* genome can be found, the expansion of this gene family may have not only predated the split between *Nicotiana* and *Solanum,* but expanded even earlier. The colocalization of *MIR* genes in *S. lycopersicum* on chromosomes 3 and 6 (supplementary fig. S4, Supplementary Material online) together with the absence of coding genes located between these *MIR* genes is the only observation consistent with a history of recent tandem duplication.

The divergence time estimates shed light on the possible sequence of duplication events. Four members of the miR482/2118 gene family (miR482a, miR482b, miR482d, and miR482g) are located within 8.2 kb on chromosome 6. The most recent ancestral node shared by these four genes is 115 Ma (fig. 3). Successive rounds of duplication subsequent to this time point gave rise to these four genes, along with the other gene family members, miR482, miR482f, miR482h, and miR5300. Because miR482, miR482f, miR482h, and miR5300 are located on chromosomes other than chromosome 6, a translocation event, followed by the tandem duplication leading to miR482 and miR482h, must be postulated to explain the current chromosomal distribution of these gene family members.

The miR482/2118 Network Shows Sequence Dependent Specialization and Evolutionary Robustness

Since the miR482/2118 gene family diversified prior to speciation of many taxa in the Solanaceae, we posed the question whether evolutionary patterns are conserved in miR482/2118 family members across species. We compared the evolutionary rates across miR482/2118 family members based on predefined structural regions of the predicted miRNA transcripts. These regions have been shown to be subject to different evolutionary constraints (Fahlgren et al. 2010). miR and miR* regions typically show the greatest constraint and hence the lowest rate of substitution, while the loop and stem regions, although important for processing, show lower constraint and hence a higher rate of substitution (Fahlgren et al. 2010). We analyzed the five regions of the miRNA genes (the miR region, the miR* region, the loop region, and the 3′- and 5′-stem regions) separately for each family member. We compared these rates to those estimated from the same plant species for the miR166 sequences. We chose the miR166 as a reference gene because it mirrored the typical substitution patterns of plant miRNAs described in Fahlgren et al. (2010), has a defined biological function and is wide-spread in the angiosperms.

Our data show that each miR482/2118 cluster has a unique evolutionary fingerprint (fig. 4). In the miR482b subclade, the stem and loop sequences are rather conserved, whereas the substitution rates in the miR and miR* regions were high. In contrast, the miR482g subclade showed little to no variation in the miR* and miR region, whereas stem and loop regions were less conserved (fig. 4). The rate of evolution of the miR region and 3′- and 5′-region were elevated in about half of the miR482/2118 clusters compared with other miRNA precursors, such as miR166 (fig. 4, Fahlgren et al. 2010). In general, at least one region had an elevated rate of substitution, except miR482.

We investigated whether these distinct evolutionary patterns correlated with a differences in target specificity across miR482/2118 genes. To do so, we predicted the targets of all miR482/2118 members in *S. lycopersicum* and *S. tuberosum* based on predicted *NBS-LRR* sequences. Although *S. tuberosum* possesses an additional miR482/2118 member, a similar proportion of *NBS-LRRs* are predicted to be targeted in both species: 19% (82 out of 434 *R*-genes) in *S. tuberosum* and 20% (52 out of 260 *R*-genes) in *S. lycopersicum* under the less stringent target prediction assumptions.

To identify general targeting patterns, we used orthoMCL to determine the *R*-gene families from the pool of predicted targets in *S. lycopersicum* and *S. tuberosum.* In general, the size of the *R*-gene family is positively correlated with the number of different miRNAs targeting the *R*-gene family ($\rho = 0.69$, P value = 0.012). In *S. lycopersicum* the predicted *R*-gene targets are more likely to be CC-NBS-LRRs, while in *S. tuberosum* the predicted *R*-gene targets are enriched for TIR-NBS-LRRs, suggesting differences in targeting between species (supplementary fig. S5, Supplementary Material online).

The total number of potential targets and the degree of redundancy in targeting varies between the miR482/2118 members of *S. lycopersicum* and *S. tuberosum.* We observe a high number of *NBS-LRR* genes that are predicted to be targets of two or more different miR482/2118 family members (fig. 5*a* and *b*, supplementary fig. S6, Supplementary

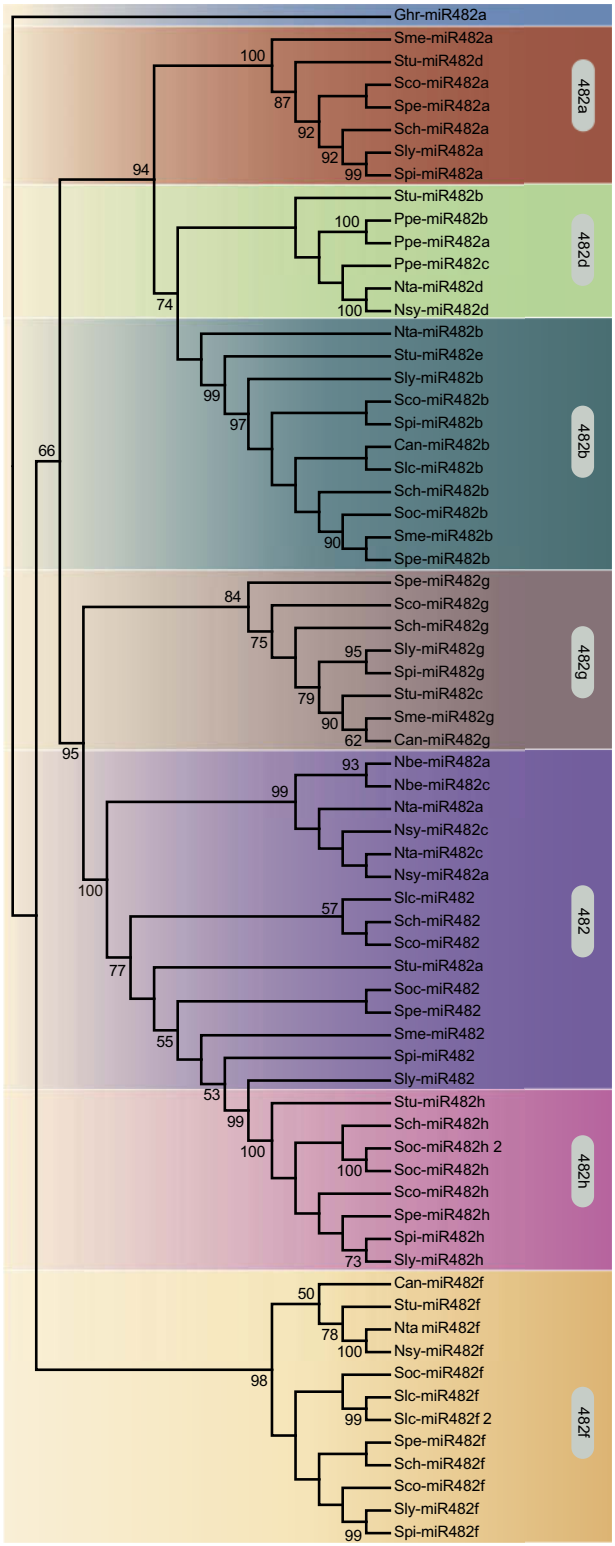

Fig. 2.—Cladogram of the miR482/2118 genes in the Solanaceae. The consensus Bayesian phylogeny of 67 miR482/2118 genes in seven miR482/2118 orthologous groups (distinguished by the different background colors). Bayesian posterior probabilities are indicated at the branches. Ghr, *Gossypium hirsutum* (outgroup); Sly, *Solanum lycopersicum*; Spi, *Solanum pimpinellifolium*; Spe, *Solanum peruvianum*; Sco, *Solanum corneliomulleri*; Sch, *Solanum chilense*; Soc, *Solanum ochranthum*; Slc, *Solanum lycopersicoides*; Stu, *Solanum tuberosum*; Sme, *Solanum melongena*; Can, *Capsicum annum*; Ppe, *Physalis peruviana*; Nta, *Nicotiana tabacum*; Nbe, *Nicotiana benthamiana*; Nsy, *Nicotiana sylvestris*. Bootstrap values greater than 50 are shown.

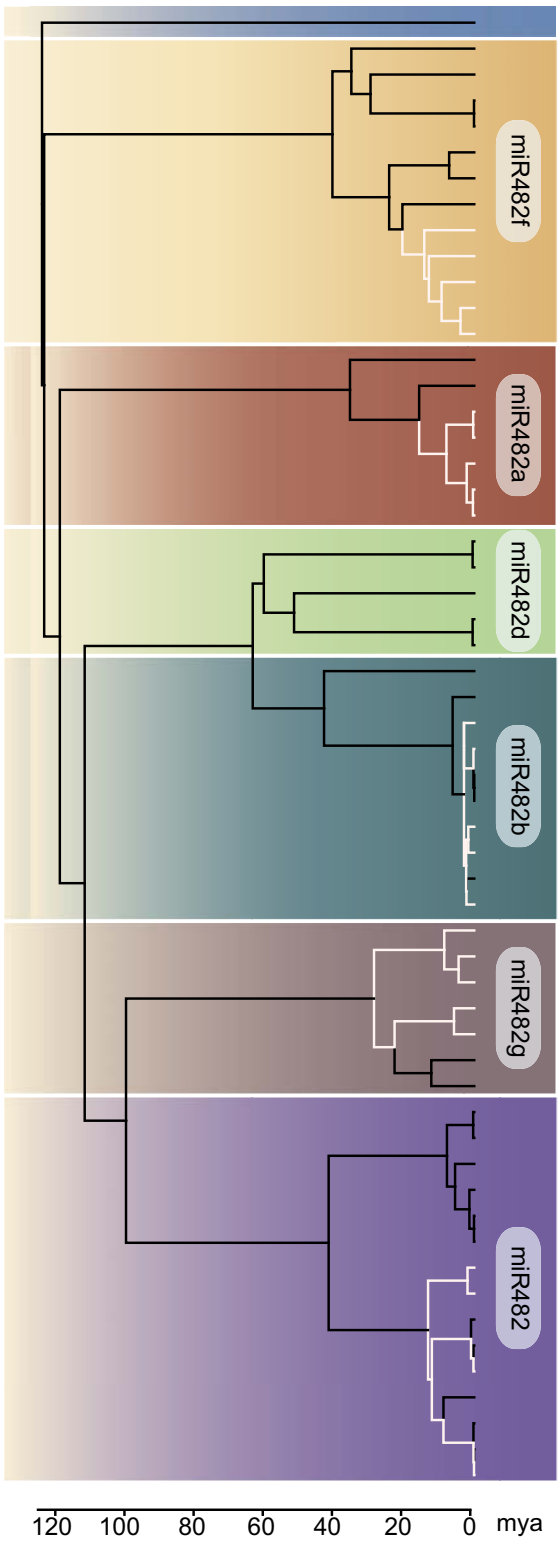

Fig. 3.—Chronogram of the miR482/2118 genes. A molecular clock was supported for a data set containing genes from six clades (distinguished by different background colors). The tree was calibrated by the rosid–asterid split (125 Ma). The miR482a gene from *Gossypium hirsutum* was used as an outgroup. The divergence times (in Ma) are above the line of the legend and the substitution rates are given below the line. The five species belonging to the wild tomato clade are indicated by white branches.

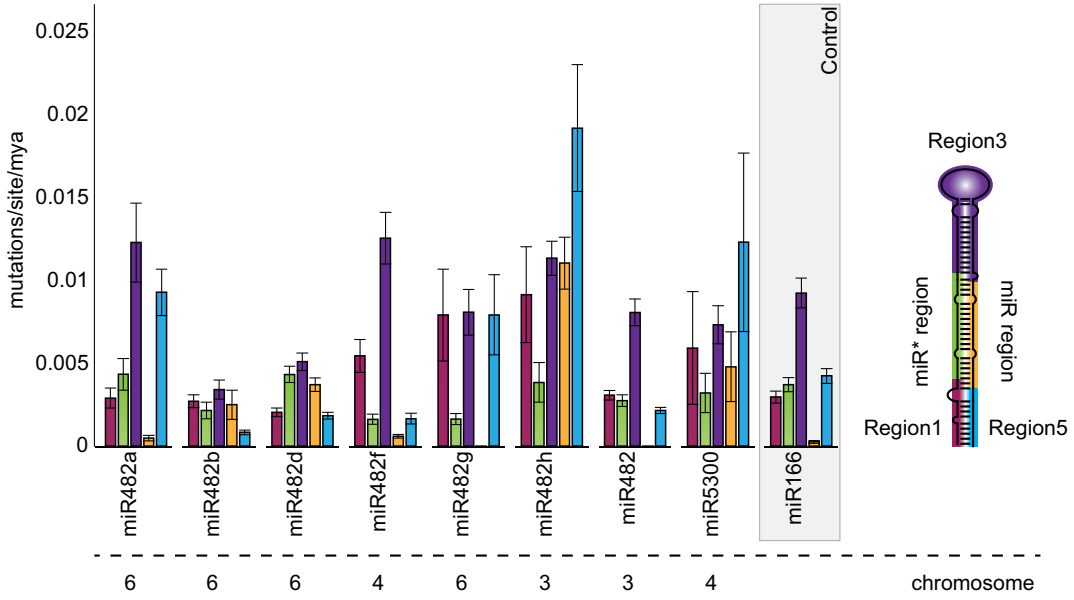

Fig. 4.—Evolutionary rates of the miR482/2118 precursor sequences. On the right, the canonical secondary structure of the miR482/2118 pre-miRNA is shown. Different coloring indicates the five functional regions of the pre-miRNA and is identical with the color coding of the bars in the histogram: 5′-region (red), miR* region (green), loop region (purple), miR region (yellow), and 3′-region (blue). miR166 is included for comparison. Below the graph, the chromosome for each gene is indicated.

Material online). All miR482/2118 family members are predicted to possess unique targets and the number of unique targets increases with the stringency of the analysis (fig. 5*a* and *b*, supplementary fig. S6, Supplementary Material online). The structurally unique miR5300 has exclusive (i.e., unique) targets only under a less stringent target prediction, and no predicted targets under strict settings (fig. 5*a* and *b*, supplementary fig. S6, Supplementary Material online).

In *S. lycopersicum*, miR482a has a high number of targets, while miR482f has only a few. These two genes, although quite different in their predicted targeting behavior, do not differ greatly in their evolutionary rates across the five regions of the *MIR* gene and their miR regions show the greatest sequence conservation (fig. 4). In general, we found no correlation between the substitution rates in the miR region and the number of targets, suggesting that the number of targets is not the only determinant of the evolutionary trajectories among members of the miR482/2118 family.

Because *R*-genes differ in their pathogen specificity, we evaluated whether certain miR482/2118 members consistently targeted *R*-genes with similar pathogen specificities (i.e., is their evidence for subfunctionalization or specialization for *MIR* genes to control specific types of *R*-genes). We observed that individual miRNAs are predicted to target multiple *R*-genes with different pathogen specificities. Therefore we concluded that the miRNAs function mainly as generalists, rather than specialists. However, the degree to which the miRNAs are connected to any particular *R*-gene family varies. We predicted clusters in the network using Fuzzifier in clusterMaker to allow for nodes (e.g., miRNAs) to be shared between *R*-gene clusters. This resulted in ten clusters, made up of one to two *R*-gene families (fig. 5*c*). Half of the miR482/2118 genes are located at the intersection between two or more clusters. If we consider the pathogen specificity of the putatively targeted *R*-genes, we find that miR482f is typically associated with *Phytophthora infestans* defense responses, miR5300 with viral defense responses and miR482a with bacterial and *P. infestans* defense responses. The remaining miR482/2118 members show a more generalist's pattern.

The observed target-dependent specialization of some miRNAs suggests that host-pathogen interactions do shape the evolution of these genes. To investigate potential evolutionary consequences of miRNA targeting for *R*-genes, we analyzed gains and losses within the predicted miRNA-target network (fig. 5*c* and supplementary fig. S7, Supplementary Material online). We observed that the paralogs within an *R*-gene family typically are predicted to be targeted by one or more of the same miRNA (86% in tomato and 80% for potato; supplementary fig. S7, Supplementary Material online). In contrast, orthologous *R*-genes (i.e., between species) are predicted to share one or more of the same miRNA only 63% of the time. Likewise, while greater than 30% of the paralogs are predicted to be targeted by exactly the same set of miRNAs, only 11% of the *R*-gene orthologs are predicted to be targeted by the exact same set of miRNAs. Therefore, targeting behavior is more strongly conserved between paralogs rather than between orthologs. Despite some

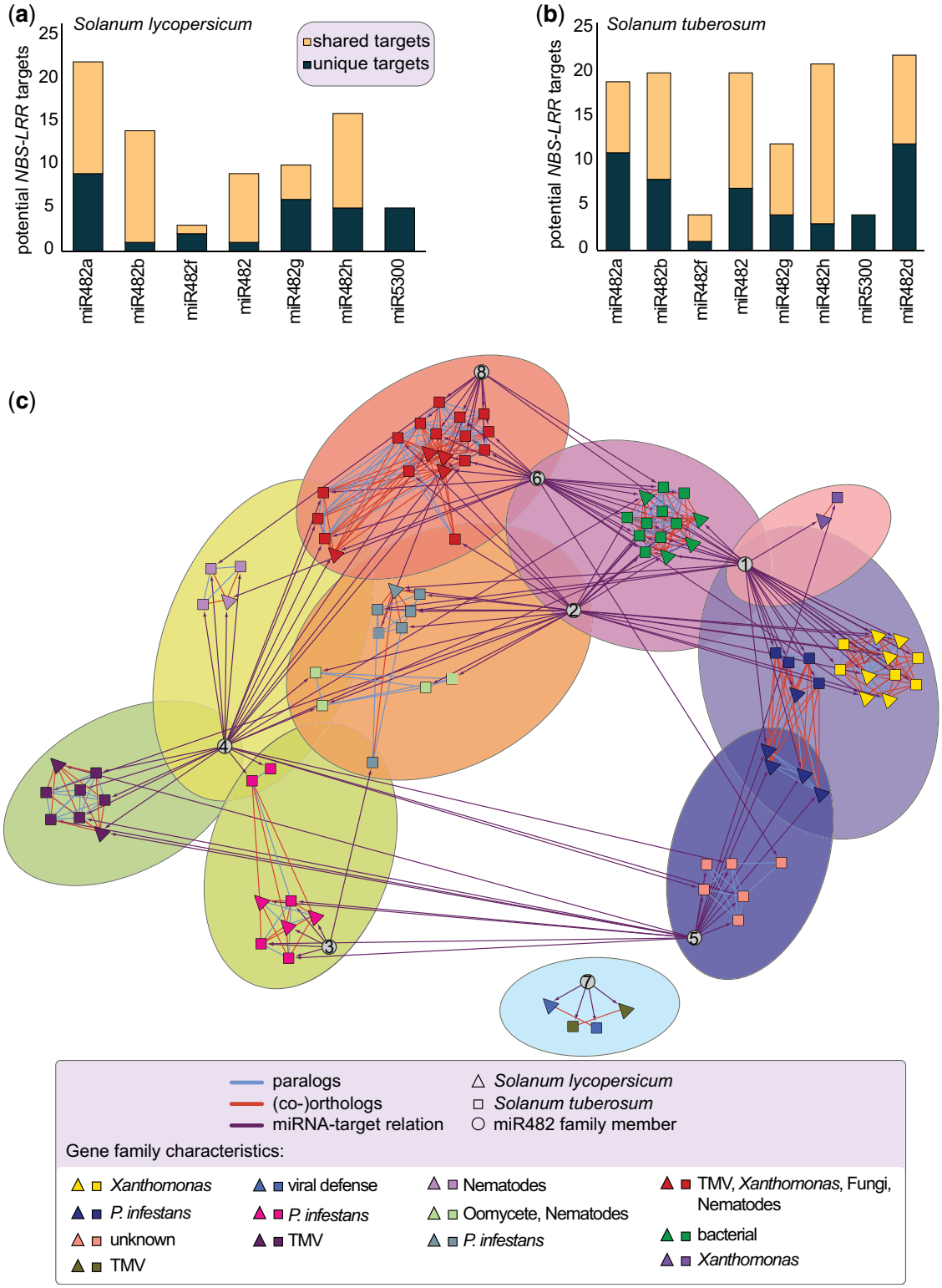

Fig. 5.—Predicted *NBS-LRR* targets of the miR482/2118 family. Unique *NBS-LRR* targets (dark turquoise) and targets shared between at least two paralogous miRNAs (yellow) in (*a*) *Solanum lycopersicum* and (*b*) *Solanum tuberosum*. (*c*) Network incorporating target predictions and evolutionary relationships between *NBS-LRRs* genes. OrthoMCL was used to determine gene families: genes from *S. lycopersicum* (triangles), genes from *S. tuberosum* (squares), orthologous relationships (orange lines), and paralogous relationships (light blue lines). Each gene family has a different color. The predicted miRNA-target relationships are indicated by direct edges (purple arrows). The miRNA nodes (gray circles) are numbered as follows: 1) miR482a; 2) miR482b;

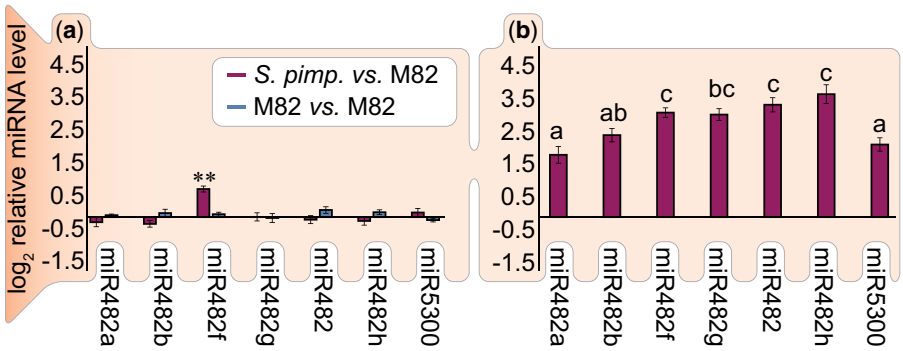

Fig. 6.—Expression of miR482/2118 in *Solanum lycopersicum* and *Solanum pimpinellifolium*. Expression levels of the miR482/2118 family members in *Solanum* species were determined using qPCR. (*a*) Expression of miR482/2118 in leaves of *Solanum pimpinellifolium* compared with *Solanum lycopersicum* cv. M82. (*b*) Comparison of expression of miR482/2118 in leaves versus roots of *S. pimpinellifolium*. Letters above the bars correspond to statistically distinct groups at P value < 0.05.

conservation, 37% of orthologous *R*-genes between *S. lycopersicum* and *S. tuberosum* are predicted to be targeted by distinct miRNAs. This reflects the potential for divergent evolution in *R*-genes that can be matched by species-specific coevolution of miRNA genes over a relatively short time span (estimated split between tomato and potato ~ 8 Ma).

Differences in miR482/2118 Expression

In addition to evaluating the evolutionary history and predicted targeting patterns, we tested if the mature miR482/2118 family members are expressed (i.e., implying correct excision from the precursor) and have species-specific or tissue-specific expression differences in the closely related species *S. pimpinellifolium* and *S. lycopersicum* using qPCR. For six mature miR482/2118 family members, no species-specific differences between *S. lycopersicum* and *S. pimpinellifolium* were detected (fig. 6*a*). However, miR482f is significantly upregulated in *S. pimpinellifolium* compared with *S. lycopersicum*.

In general, all mature miR482/2118 members have higher expression in leaves compared with roots (fig. 6*b*). Three statistically significantly different expression classes for these seven miRNAs could be detected: miR482, miR482f, and miR482g had the greatest upregulation, whereas miR5300 and miR482a had the least. The three miRNAs, miR482, miR482f, and miR482g, with the greatest upregulation share a number of predicted *R*-gene targets (figs. 5*c* and 6*b*). This indicates that expression patterns may correlate with the target profile. A surprising result was that miR482a and miR482g had different pattern of expression, but lie within 200 bp of one another in the genome. Differential transcript abundance may arise from differential processing of the pri- or pre-miRNAs. This physically closest pair of genes shows not only expression differences of the corresponding miRNAs, but also differences in targeting. This underscores the observation that despite redundancy in the predicted target network (i.e., targeting of the same *R*-gene by multiple miRNAs), some target specialization has arisen.

Discussion

The miR482/2118 gene family is a negative regulator of plant resistance genes (Zhai et al. 2011; Li et al. 2012). It is an evolutionarily striking family due to its presence/absence variation throughout the plant kingdom and its high within and between species sequence variation of mature miRNA sequences. To better understand the evolutionary history of this gene family, we studied these genes in the Solanaceae with a focus on wild tomatoes.

miR482/2118 Is an Ancient miRNA Family

Within the Solanaceae, we detected eight different genes belonging to the miR482/2118 gene family in the 14 species analyzed. In agreement with the study of Shivaprasad et al. (2012), we observed high variation in the mature miRNA sequences between species (fig. 4). However, the use of entire precursor sequences allowed us to identify orthologs between species, despite differences in their mature miRNA regions.

The miR482/2118 gene family repertoire of the nightshades diversified prior to speciation within the Solanaceae. Some duplication events appear to have occurred more than 100 Ma, corresponding to the split between the Phrymaceae, the family to which *Mimulus* belongs, and the Solanaceae.

Fig. 5.—Continued
3) miR482f; 4) miR482; 5) miR482g; 6) miR482h; 7) miR5300; and 8) miR482d. The nodes were clustered with Fuzzyfier according to connectivity, allowing shared clusters. Clusters are shown by the different background colors. 50% of miRNAs share clusters. Pathogen specificities of the different *R*-gene families are given in the legend.

While sequences related to miR482/2118 can be found in the *M. guttatus* genome, they do not appear to fold, indicating subsequent gene loss or degeneration in this species.

Although it was not possible to reconstruct the ancient large-scale duplication events, we could identify younger tandem duplications between miR482a, miR482b, miR482d, and miR482g on chromosome 6 and miR482 and miR482h on chromosome 3. Based on our observations, we hypothesize that miR482/2118 is an ancient miRNA family that radiated early on in plant evolution involving large-scale and tandem duplication events. The observed variation in the mature miRNA regions of orthologous miR482/2118 genes between even closely related species (e.g., within *Solanum*) is best explained by species-specific miR482/2118 repertoire and its coevolution with its targets, rather than convergent evolution leading to a unique miR482/2118 inventory in each species, as previously suggested (Shivaprasad et al. 2012). A recent study of this gene family in the gymnosperm species, *Pi. abies*, indicates that the gene family originated following the split between ferns and Gymnosperms (Xia et al. 2015).

miR482/2118 Homologs Show Unique Evolutionary Fingerprints

R-genes are typically dynamic and fast evolving components of plant genomes (Clark et al. 2007). However, within a given *R*-gene family, evolutionary rates can differ among paralogs, with some showing rapid evolution and others evolving more slowly (Kuang et al. 2004). We see an analogous situation for the miR482/2118 family in the Solanaceae.

Evolutionary rates are predicted to be lower for taxonomically widespread miRNAs maintained over long evolutionary timescales (so-called ancient or conserved miRNAs) than for miRNAs of more recent origin (Fahlgren et al. 2010; Meunier et al. 2013). miR166 is one such conserved miRNA and helps to control root development (Carlsbecker et al. 2010; Fahlgren et al. 2010). In contrast, the miR482/2118 family, although widespread and ancient (Xia et al. 2015), departs from this pattern. Members of this gene family show an elevated rate in at least one region of the precursor sequence. Coevolution with their *R*-gene targets, which themselves show elevated sequence evolution, may contribute to differences in evolutionary history compared with more conserved miRNAs.

miR482/2118 Targeting Is Highly Dynamic and May Function as a Buffer in R-Gene Evolution

miR482/2118 family members are predicted to target a large subset of *R*-genes. Despite their differences in evolutionary rates, these miRNAs appear to be mainly generalists based on the pathogen spectrum of their potential *R*-genes targets. To investigate this paradox, we explored the predicted *R*-gene–miRNA targeting relationships in more detail. We observed that despite several unique predicted targets, nearly all miRNA genes share some predicted targets. This results in a highly interconnected network, in which larger *R*-gene families are predicted to be targeted by more miRNAs compared with smaller *R*-gene families. A correlation between *R*-gene family size and number of targeting miRNAs has been observed in other species (González et al. 2015). Hence, this robustness seems to be conserved in evolution and is potentially an important feature of *R*-gene regulation.

Fei et al. (2013) put forth the hypothesis that miRNAs act as buffers for *R*-genes. They argued that multiple layers of regulation may be evolutionarily beneficial since mutations introduced into *R*-gene promoters could lead to autoimmune responses and thus high fitness costs for the plant. Another hypothesis put forward by Li et al. (2012) and Shivaprasad et al. (2012) is that *R*-gene targeting by small RNAs help to support *R*-gene evolution. They suggest that transcriptional suppression could dampen fitness costs of poorly functioning *R*-genes and thus relax constraints on *R*-gene sequences; ultimately leading to *R*-gene diversification, sub- and neofunctionalization. These two hypotheses are not mutually exclusive, and in fact, can be united. We show that *R*-genes in *S. lycopersicum* and *S. tuberosum* are typically predicted to be targeted by two or more miR482/2118 members. This redundancy likely protects against the negative consequences of misexpression of *R*-genes. It may also allow for cryptic genetic variation to accumulate at *R*-genes.

We also observed that fewer orthologous *R*-genes between *S. lycopersicum* and *S. tuberosum* are predicted to be targeted by the same miRNA compared with paralogs within species. This may arise from species-specific *R*-gene duplications and divergence subsequent to speciation (i.e., independent diversification of *R*-gene families following speciation). Therefore, divergence in targeting may have been concomitant with changes in *R*-gene repertoire after these species split. This may be advantageous to continue to maintain the appropriate suppression of targets. All in all, this points to a high buffering capacity of the targeting network, in which potential losses of targeting relationships are off-set by the high interconnectivity of the network, despite ongoing subfunctionalization of the miRNAs.

Supplementary Material

Supplementary figures S1–S7 and tables S1 and S2 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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10.2 Publication II: Signatures of selection and host adapted gene expression of the *Phytophthora infestans* RNA-silencing suppressor PSR2

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Major – initiated and designed the study, performed parts experiments, analyzed and interpreted the data, prepared most figures and drafted the manuscript.

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Signatures of selection and host adapted gene expression of the *Phytophthora infestans* RNA-silencing suppressor PSR2

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Summary

Phytophthora infestans is a devastating pathogen in agricultural systems. Recently an RNA silencing suppressor (PSR2) was described in *P. infestans.* PSR2 has been shown to increase the virulence of *Phytophthora* pathogens on their hosts. This gene is one of the few effectors that is present in many economically important *Phytophthora* species. In this study, we investigated i) the evolutionary history of *PSR2* within and between species of *Phytophthora* and ii) the interaction between sequence variation, gene expression and virulence. In *P. infestans*, highest Pi*PSR2* expression was correlated with decreased symptom expression. Highest gene expression was observed in the biotrophic phase of the pathogen, suggesting that PSR2 is important during early infection. Protein sequence conservation is negatively correlated with host range, suggesting host range as a driver of PSR2 evolution. Within species we detected elevated amino acid variation, as observed for other effectors; however the frequency spectrum of the mutations is inconsistent with strong balancing selection. This evolutionary pattern may be related to the conservation of the host target(s) of PSR2 and the absence of known corresponding *R* genes. In summary, our study indicates that PSR2 is a conserved effector that acts as a master switch to modify plant gene regulation early during infection for the pathogen's benefit. The conservation of PSR2 and its important role in virulence makes it a promising target for pathogen management.

Introduction

Phytophthora infestans is one of the most devastating plant pathogens and infects many crop species of the Solanaceae, including potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). *Phytophthora* species are hemibiotrophic pathogens, having a biotrophic followed by a necrotrophic phase. During this biotrophic stage the hyphae never invade the plant's cells, but acquire nutrients from their host using haustoria (Blackwell, 1952; Allen and Friend, 1983; Voegele et al., 2001). In the necrotrophic phase the pathogens grow intracellularly and lyse the host cells to live on degraded compounds (van Kan et al., 2006). During the infection cycle, *Phytophthora* spp. employ different

strategies to circumvent plant defense. In the first phase, *Phytophthora* spp. modulate the host immune system to suppress host-induced cell death (Kelley et al., 2010). In the second phase, host cell death is promoted by the pathogen (Vleeshouwers and Oliver, 2014).

As with other plant pathogens, pathogenicity and virulence are determined by effector genes (Oh et al., 2009; Lo Presti et al., 2015). Effector proteins are secreted into the host cytoplasm and may transit to the host nucleus where they modulate plant signaling and the plant's immune responses (Whisson et al., 2007; Dou et al., 2008; Schornack et al., 2010). Genome-sequencing of several *Phytophthora* species uncovered two major types of effector proteins in this pathogen lineage: the RxLR-effectors, identified by the amino acid (aa) motif RxLR in the N-terminus of the protein sequences (Rehmany et al., 2005) and the Crinkler-effectors (CRN) (Torto et al., 2003). Genome-wide comparison of many *Phytophthora* species showed large expansions of RxLRs and CRNs within these species (Tyler et al., 2006; Jiang et al., 2008; Haas et al., 2009; Stam et al., 2013). Therefore, RxLRs and CRNs show high rates of gains and losses between species (Haas et al., 2009).

In addition to presence/absence variation, effectors typically show high rates of nucleotide substitution (Allen et al., 2004; Rehmany et al, 2005; Allen et al., 2008; Raffaele et al., 2010). In fact at some genes, the rate of non-synonymous substitutions (changes that lead to aa differences) are even higher than the rate of synonymous substitutions (changes that do not lead to aa differences), suggesting that effectors are subjected to balancing selection (Allen et al., 2004; Rehmany et al., 2005; Oliva et al., 2015). In addition to harboring sequence variation at functionally important effector loci, some pathogens show differential expression. Cooke et al. (2012) found that the highly aggressive *P. infestans* isolate 06_3928A expresses 1013 more genes than the weak isolate T30-4 during infections on potato. Likewise, Qutob et al. (2013) show transgenerational silencing of effector genes to avoid host detection. Taken together, the presence of copy number variation, high substitution rates, signatures of balancing selection and differential expression of effectors reveal the dynamic nature of pathogen adaptation.

The need to constantly avoid host detection and defense results in little overlap of the effector repertoire between species or even strains: In a comparison of three oomycete genomes, only four universal effector genes were found (Win et al., 2007). This represents 0.6% to 2.7% of the effectors known in these species. The conservation of this small number of effectors likely reflects their essential role in virulence. One such effector, "*Phytophthora* suppressor of RNA silencing 2" (PSR2), has recently been characterized (Qiao et al., 2013). PSR2 homologs were detected in different *Phytophthora* species and even in *Hyaloperonospora arabidopsidis* (Xiong et al., 2014). As revealed by its name, PSR2 suppresses RNA silencing in the different hosts of *Phytophthora* spp. (Qiao et al., 2013; Xiong et al. 2014).

Small RNAs (sRNAs) are essential for development and defense of plants (Carlsbecker et al., 2010; Bocarra et al., 2014); therefore, manipulation of sRNA production can negatively affect a plant host. Mutants of the sRNA biogenesis machinery display severe developmental phenotypes (including lethality) or impairment in disease resistance against several pathogens (Hendelman et al., 2013; Kravchik et al., 2014; Pumplin and Voinnet, 2013). Also, microRNAs (miRNAs) involved in resistance regulation have been identified as potential contributors to host adaptation (de Vries et al., 2015). Targeting this machinery may therefore be a means to undermine host resistance.

sRNA can be formed in two related processes. In the primary step double stranded RNA (dsRNA) is processed by Dicer-like proteins (DCLs) into double stranded small interfering RNAs (siRNAs), or primary miRNA (pri-miRNA) transcripts are processed into double stranded miRNAs (Kurihara and Watanabe, 2004; Rogers and Chen, 2013). siRNAs and miRNAs will be incorporated into Argonaute proteins (AGOs) (Rogers and Chen, 2013). The loaded AGOs direct the sRNAs to complementary sequences of mRNA where they form small stretches of dsRNA, which initiate the mRNA degradation by the AGOs (Rogers and Chen, 2013). In the secondary biogenesis step the mRNA is not fully degraded by the AGOs, but cut in a specific pattern: RNA-dependent RNA polymerase 6 (RDR6) recreates a dsRNA from the mRNA target, which is further processed by DCL4 again into siRNAs (Axtell

et al., 2006; Chen et al., 2010; Cuperus et al., 2010). These siRNAs are usually generated in a 21nt phased pattern and are hence called phased small interfering RNAs (phasiRNAs) (Allen et al., 2005; Yoshikawa et al., 2005; Zhai et al., 2011).

Expression of *PSR2* in *Arabidopsis thaliana* led to a specific reduction in phasiRNAs (Qiao et al., 2013). Both Qiao and colleagues (2013) and Xiong and colleagues (2014) showed that overexpression of *PSR2* enhances *Phytophthora* infection in several hosts and is therefore important for the virulence of oomycete pathogens. While the exact mechanism by which PSR2 downregulates phasiRNAs remains unclear, these findings suggest that PSR2 targets an essential, ubiquitous protein of the plant RNA silencing machinery; a target shared across many potential host species may explain the widespread conservation of PSR2 across species of *Phytophthora*.

To better understand the evolutionary history of PSR2, we investigated this gene in the late blight pathogen *P. infestans* using well-established population genetic methods. We also evaluated the expression of the *P. infestans* PSR2 gene (Pi*PSR2*) across different isolates during infection of *S. lycopersicum* and its closely related sister-species *Solanum pimpinellifolium*. Although the presence of *PSR2* is conserved at the species and isolate level, the PSR2 protein evolves in a lineage-specific manner. We found that *Pi*PSR2 has significantly elevated rate of aa polymorphism in *P. infestans* compared to PSR2 in *Phytophthora sojae* (*Ps*PSR2). Nevertheless, Pi*PSR2* still shows signatures of purifying selection, in agreement with a conserved host target. Furthermore we found that Pi*PSR2* is expressed during the biotrophic phase, suggesting a role in establishing the infection. Further, symptom development is negatively correlated with expression of Pi*PSR2.* We hypothesize that PSR2 acts as a master modulator of plant signaling to disrupt plant physiology at the site of infection.

Results

Pi*PSR2* is widespread across species of *Phytophthora*.

PSR2 is a rare example of an effector gene that is present in many plant pathogens of the peronosporalien oomycete lineage (Win et al., 2007; Xiong et al., 2014). However, this does not preclude the possibility of presence/absence variation within species. To determine whether the presence of this gene is also conserved below the species level, we surveyed 19 isolates of *P. infestans* from Europe, the Americas and Africa combining traditional molecular methods and genome analyses. We detected the Pi*PSR2* gene in all isolates. Furthermore, all detected Pi*PSR2* sequences were full-length compared to the reference sequence and no premature stop codons were found. Additionally, we evaluated four *P. sojae* isolates (Wang et al., 2011) for the presence of Ps*PSR2* and found that all tested isolates possessed the full-length gene. In our analysis of five well-characterized datasets of *Phytophthora parasitica* isolates, we detected a recent duplication of the *P. parasitica PSR2* gene (Pp*PSR2*) after the split from *P. parasitica* and *P. infestans* (Figure S1). All *P. parasitica* isolates possessed at least one *PSR2* duplicate.

Differences in protein variation across species.

Given that *P. parasitica* showed recent gene duplication of PSR2, we hypothesized that PSR2 may evolve in a lineage-specific manner. Therefore, we evaluated the sequence diversity of alleles within three *Phytophthora* species. Alleles of PSR2 from different isolates of *P. sojae* were 100% identical at the protein level. Alleles of *P. infestans* were 99.4 % identical at the protein level, while the alleles of *P. parasitica* were 95.3% identical for copy A and 92.8% identical for copy B proteins.

Despite maintenance of the *PSR2* gene, amino acid polymorphism is elevated within species.

We evaluated the sequence variation within Pi*PSR2* in 16 of the 19 isolates of *P. infestans.* The N-terminus encoding the signal peptide and the translocation motif had a higher average pairwise nucleotide diversity (π) than the full-length gene or the C-terminus alone (Table 1). To determine whether the patterns of sequence variation at *PSR2* are typical for these isolates, we analyzed the sequence variation in a subset of six to eight isolates at three reference genes: *Elongation factor 1*α

(Pi*Elf1α*), *ATP Synthase subunit δ* (Pi*ATP5D*) and *Argonaut1* (Pi*AGO1*). Pi*AGO1* had the lowest overall π of the three genes, while Pi*AT5D* had the lowest non-synonymous variation (π_a = 0.00; Table 1). Relative to these reference genes, π_a is elevated for Pi*PSR2* in both the 16 and the eight isolate set. Furthermore Pi*PSR2* has the highest number of segregating sites and a high ratio of π_a to π_s. While the ratio of π_a/π_s across the entire gene is not greater than 1, π_a/π_s is higher at Pi*PSR2* than at Pi*ATP5D* and Pi*Elf1α*. Furthermore, although the ratio of π_a/π_s for Pi*AGO1* is similar to Pi*PSR2*, Pi*AGO1* has few segregating sites (4) compared to Pi*PSR2* (27).

An elevated π_a/π_s ratio can be an indication of either relaxed selective constraint or balancing selection maintaining distinct protein variants. Therefore, we conducted a McDonald-Kreitman test (McDonald and Kreitman, 1991) to determine if the nucleotide patterns differed from the expectations under neutrality (Tables 1, S1). According to this test, the variation at Pi*PSR2* deviates from neutral expectations: Replacement polymorphisms within *P. infestans* at the Pi*PSR2* are significantly elevated relative to the expectation under neutrality (Tables 1, S1). To better visualize the evolution of this gene within *P. infestans*, we mapped the aa changes of PSR2 onto the phylogeny of nine isolates for which the complete mitochondrial genome was available (Figure S2). Nearly all protein substitutions map to the tips of the mtDNA tree and the accumulation of aa differences in PSR2 does not correlate with the branch lengths of the mtDNA tree (Figure S2).

Amino acid variation across *Pi*PSR2 secondary structure.

To better understand how the protein variation was distributed both across the *Pi*PSR2 protein and across individuals of this species, we evaluated the location of the aa polymorphisms on the predicted secondary structure of *Pi*PSR2. The overall secondary structure is conserved across the *Pi*PSR2 alleles of *P. infestans*; all alleles were predicted to contain an N-terminal signal peptide and 40 consecutive α-helices (Figure 1). Of the aa polymorphisms, 45% lie within a predicted α-helix (Figure 1). Among the 20 observed aa polymorphisms, 14 encoded radical changes, involving differences in polarity, charge or aa structure. Of those 14 radical changes, five involved proline residues, which can have a

strong effect on protein structure. Since PSR2 was suggested to affect a step in the sRNA biogenesis that takes place in the cytosol (Qiao et al. 2013, Bologna and Voinnet 2014) and hydrophobic residues generally reduce protein solubility in water, we investigated the distribution of hydrophobic residues in *Pi*PSR2. Approximately 42% of the residues in the T30-4 reference allele were hydrophobic. Of the 20 polymorphic positions, 12 involved a hydrophobic residue (35%, Figure 1). Seven of these polymorphisms were radical changes. With the exception of L283S, each of these radical changes was singleton polymorphism in our sample (i.e. the substitution was only found in one individual).

We identified several mutations that are radical and ought to have an influence on protein structure. Yet the overall protein structure was predicted to be conserved across all isolates. Effectors of oomycetes often have W- and Y-motifs in their protein sequence (Jiang et al., 2008; Boutemy et al., 2011). These motifs confer a certain robustness to the protein structure allowing for adaptive mutations to occur within them without compromising the protein functionality (Jiang et al., 2008; Boutemy et al., 2011). Ye and Ma (2016) identified that PSR2 homologs possess several W- and Y-motifs. Here, we manually searched for these motifs and predicted motifs using MAST. We found 16 W-like sequences and 17 Y-like sequences in *Pi*PSR2. However, only seven sequences per motif contained 50% or more of the residues previously described in Jiang et al. (2008) and Boutemy et al. (2011) (Figure 1). The seven W-like motifs were also identified by three MAST motifs (Figure S3), suggesting a total of three W-motif-types. The remaining W-like sequences were distributed over two to three MAST motifs. Six out of seven Y-like motifs were located within a few aa positions following the W-like motifs or partially overlapped with the W-like motifs, in agreement with what was observed by Boutemy et al. (2011). All Y-like-motif-types were either distributed over two MAST motifs or present nearly completely in one MAST motif (Figure S3). We only considered the conservative W- and Y-motifs for further analyses. Six aa polymorphisms mapped to four W-motifs and three aa polymorphisms mapped to two Y-motifs. One polymorphism was shared between a W- and Y-like motif. In total 40% of the aa polymorphisms mapped to either a W- or Y-motif.

Relationship between symptom strength and Pi*PSR2* sequence.

To test whether allelic variation of the Pi*PSR2* gene is associated with the strength of symptoms caused by *P. infestans* on cultivated tomato, we conducted inoculations of seven *P. infestans* isolates on *S. lycopersicum* cv. Moneymaker and evaluated the symptom strength after three days post inoculation (dpi). Symptom strength was quantified by calculating the relative leaf area covered in necrotic lesions following inoculation. The ranking of the isolates based on symptom strength was: NL10001/NL88069<T20-2<NL90128<IPO-C/T15-2/D12-2 (Figure 2a). No correlation between symptom strength and allele sequence of *Pi*PSR2 was detected (Figure 2b).

Relationship between symptom development and Pi*PSR2* expression.

While multiple protein variants of *Pi*PSR2 segregate in *P. infestans* and isolates vary in their ability to produce symptoms on tomato, protein variation alone does not explain the variation in virulence. In addition to primary sequence, differences in gene expression may also contribute to pathogen virulence. To determine whether Pi*PSR2* gene expression affects virulence, we performed inoculations on two closely related host species (*S. lycopersicum* and *S. pimpinellifolium*) with weak and strong isolates of *P. infestans*. Based on our previous experiment, T20-2 was chosen as a weak isolate and IPO-C and D12-2 as strong isolates (Figure 2b). Pathogen abundance (evaluated by molecular markers) and the extent of the relative necrotic area was determined at multiple time points. In general, all isolates showed an increase in the relative necrotic area over time in both hosts, and this correlated with pathogen abundance as assayed by qRT-PCR (Figures 3, S4, S5). We also noticed that IPO-C caused slightly more symptoms than D12-2. However, the hosts differed in their specific responses to these pathogens. The pattern of the necrotic lesions were dispersed around the veins in *S. lycopersicum,* while the lesions were concentric around the point of inoculation in *S. pimpinellifolium* (Figures 3a, S4). Also *S. lycopersicum* had a larger relative necrotic area than *S. pimpinellifolium* in infections with the strong isolates*,* while *S. lycopersicum* had a smaller necrotic area than *S. pimpinellifolium* in infections with the weak isolates (Figures 3a, 3b, S5).

To determine whether a lower proportion of necrotic area at a given time point corresponded to a delay in the pathogen's transition from biotrophy to necrotrophy, we analyzed the presence of important anatomical structures, such as haustoria and sporangia, across samples (Figures 4a-f). Haustoria are present early in infections and continue to increase as the pathogen spreads. Nevertheless, at 96 hours post inoculation (hpi) a reduction of haustoria is visible for some infections (Figures 4g, S6a). Sporangia appear later in infection and indicate the transition to necrotrophy. In infections with the strong isolate, IPO-C, on both hosts, haustoria were detected at all time points. By 72 hpi each assayed leaflet had at least one haustorium (Figure 4g). Sporangia occur first at 72 hpi in all infections (Figures 4g, S6). We therefore infer that the transition of IPO-C from biotrophy to necrotrophy is between 48 hpi and 72 hpi. Semi-quantitative RT-PCR analyses of marker genes specific for the biotrophic and necrotrophic phase are consistent with this time of transition (Figures 4i, 4h). The other strong isolate, D12-2, showed a pattern similar to IPO-C, although fewer leaflets contained haustoria in both hosts at each time point and fewer leaflets of *S. pimpinellifolium* had sporangia at 72 and 96 hpi (Figure S6a), indicating a delay in transition in some leaflets of *S. pimpinellifolium*. For the weak isolate, T20-2, there were fewer haustoria at 48 hpi compared to the strong isolates (Figure S6b). These haustoria were also immature, while the strong isolates produced many more mature haustoria from 48 hpi onwards. Also only few sporangia were detected at 72 hpi and 96 hpi. This shows that the weak isolate, T20-2, has a delayed development and transition from biotrophic to necrotrophic phase in both hosts.

The earliest detection of Pi*PSR2* expression was from 24 hpi onwards; i.e. when the infection was fully established. The average CT-values over time for Pi*PSR2* were 34.32 1.65 for the weak isolate (T20-2) and 30.69 2.23 for IPO-C and 31.56 2.62 for D12-2, the strong isolates. These values indicate that Pi*PSR2* has moderately low expression during infection and suggests that the weakest isolate has the lowest Pi*PSR2* expression. However, since biomass of the three isolates varied, the expression of Pi*PSR2* was normalized with expression of the biomass markers, Pi*H2a* (*Histone 2a*) and Pi*Elf1α*. Following appropriate normalization against the biomass markers, we found that at most time points

D12-2 and T20-2 had significantly higher relative expression levels compared to IPO-C in both hosts (Figure 5). Additionally, T20-2 often had significantly higher expression levels than D12-2 (Figure 5). This negatively correlates with the extent of symptoms caused by the isolates, suggesting that higher Pi*PSR2* expression is associated with reduced virulence (Figures 3, 5, S5).

To test whether a correlation between expression and symptom strength is present for other effectors, we analyzed the expression of a *CRN* effector gene, *PITG12646*, in these isolates (Figure S7). At nearly all time points, *PITG12646* shows the highest expression in the strong isolate D12-2, while expression of *PITG12646* was lower and similar in the other two isolates. Therefore, the correlation between symptom strength and expression is specific to the effector assayed.

Pi*PSR2* is expressed during biotrophy.

Effectors can be expressed constitutively throughout infection or in a life stage dependent manner (Judelson et al., 2008; Jupe et al., 2013). Therefore we hypothesized that Pi*PSR2* expression may differ between isolates that show different transition times and symptom development. The patterns of Pi*PSR2* expression depended both upon pathogen isolate and host genotype (Figure 6). For each pathogen strain, differences in Pi*PSR2* expression across time points were not detected, indicating more or less constant expression from 24 hpi onwards (Table S2). The only exception was IPO-C, for which Pi*PSR2* expression was significantly down regulated in both hosts at 72 relative to 24 hpi (Table S2). This indicates that the Pi*PSR2* expression is highest in the biotrophic phase of IPO-C and decreases as the pathogen transitions into necrotrophy, while in the other pathogen strains, Pi*PSR2* expression stays constant during the infection. For IPO-C and T20-2, the relative expression of Pi*PSR2* over time is negatively correlated with biomass (IPO-C-*S. lycopersicum* ρ=-0.68, p-value=$4.341*10^{-6}$; IPO-C–*S. pimpinellifolium* ρ=-0.67, p-value=$6.986*10^{-6}$; T20-2-*S. lycopersicum* ρ=-0.87, p-value=$3.009*10^{-11}$; T20-2–*S. pimpinellifolium* ρ=-0.78, p-value=$1.450*10^{-8}$).

Isolate-specific and host-specific effects on Pi*PSR*2 expression.

On *S. lycopersicum*, Pi*PSR2* expression is significantly different between IPO-C compared to either D12-2 or T20-2 (Figure 6a, Table S2). While on *S. pimpinellifolium* Pi*PSR2* expression is significantly different between D12-2 compared to either IPO-C or T20-2 (Figure 6b, Table S2). In summary, depending on the pathogen and host combination, Pi*PSR2* expression is either elevated in the biotrophic phase or constant over time, but never elevated during the transition from biotrophy to necrotrophy or during the necrotrophic phase.

Discussion

Pi*PSR2* evolves under purifying selection.

PSR2 is one of the two recently identified oomycete RNA silencing suppressors (Qiao et al., 2013). The presence and function of PSR2 is conserved across many species of *Phytophthora* (Xiong et al., 2014). Overexpression of *PSR2* enhances virulence of *Phytophthora* spp. independent of the pathogen-host system (Xiong et al., 2014). These observations, along with our detection of full-length alleles within all *P. infestans* isolates analyzed, underscore the likely biological importance of PSR2 as a virulence factor in *Phytophthora*. Therefore, the observed elevated rate of aa evolution within *P. infestans* is at odds with the strong functional conservation at higher taxonomic levels. Elevated protein polymorphism relative to divergence (as observed at Pi*PSR2*) can be observed under two contrasting evolutionary scenarios: Either the protein is evolving under relaxed selective constraint or natural selection maintains alternative protein variants (Li 1997). Under the latter scenario of balancing selection, polymorphisms are typically maintained at intermediate frequency in the population. The presence of many singleton mutations observed at Pi*PSR2* is therefore inconsistent with predictions of balancing selection. However, the fact that π_a/π_s is still <1 indicates that the gene is not evolving completely devoid of selective constraint. Furthermore, all alleles, despite their aa differences, are predicted to encode a common secondary structure made up of consecutive alpha helices, and Pi*PSR2* was expressed in every isolate tested. These observations point towards the presence of continued functional constraint on the gene, despite the high rate of aa evolution. One possible

explanation is that certain structurally conserved features of *Pi*PSR2 compensate for the accumulation of additional aa changes. For example, *Pi*PSR2 contains multiple W- and Y-motifs, also present in other effector molecules (Jiang et al., 2008; Boutemy et al., 2011). The W- and Y-motifs provide structural integrity to an effector protein (Boutemy et al., 2011). Hence, the presence of these motifs in *Pi*PSR2 could provide robustness and thus release the selective constraint at other positions in the molecule. In agreement with this, Jiang and colleagues (2008) showed that the W- and Y-motif are enriched for adaptive mutations. Since *P. infestans* has a broad host range, these alternative protein variants may contribute to virulence on different hosts. However, based on our analysis of two closely related tomato species, no relationship between symptom strength and *Pi*PSR2 sequence was detected. Based on these observations, we conclude that purifying selection has likely dominated the evolution of Pi*PSR2* and that the elevated protein polymorphism within *P. infestans* could be related to specific features of this pathogen, such as its wide host range and nearly world-wide distribution. The within-species protein variation of *Ps*PSR2, *Pp*PSR2 and *Pi*PSR2 further support the role of host range in PSR2 evolution: All *Ps*PSR2 sequences were identical and *P. sojae* has the smallest host range, with only soybean as its host (Kuan and Erwin, 1980; Pratt, 1981; Crawford et al., 1996). *Pi*PSR2 showed more variation than *Ps*PSR2 and *P. infestans* can infect many Solanaceae (Adler et al., 2002; Flier et al., 2003; Garry et al., 2005). *P. parasitica* has the broadest host range of these three species (72 plant species from different families; Hickman, 1958). *Pp*PSR2 had not only the highest variation within the protein, but also showed a recent duplication after the split of *P. parasitica* and *P. infestans.* Hence it is possible, that host range is a driver of *PSR2* evolution.

Variation in expression Pi*PSR2* is correlated with virulence.

Although sequence variation in *P. infestans* at the Pi*PSR2* gene does not correlate with virulence in the cultivar *S. lycopersicum* cv. Moneymaker, expression variation was correlated with symptom development. We found that Pi*PSR2* peaks early during the biotrophic phase and decreases as *P. infestans* biomass increases. Downregulation of Pi*PSR2* was the greatest in the most aggressive

strains. This is in contrast to the findings of Xiong et al. (2014). In that study, transient overexpression of *PSR2 in planta* increased virulence of several *Phytophthora* species on their hosts. One possible explanation for these contrasting observations could be that while high expression is advantageous *in planta*, it may not be advantageous within the pathogen itself. It is possible that high expression of *Pi*PSR2 could lead to off-targeting within the pathogen. In a different study, Wang et al. (2011) reported that aberrant expression of effector genes (either too high or too low) by the pathogen decreases pathogen virulence.

The change in expression over time is not only correlated with virulence, but is also pathogen and host dependent. In *P. sojae*, Ps*PSR2* has its highest expression when *P. sojae* enters the transition phase (Xiong et al., 2014), while Pi*PSR2* shows either constant expression over time or elevated expression in the early biotrophic phase. However, we never observe an upregulation of the effector in the necrotrophic phase. Isolate-specific differences in effector gene regulation could be due to self-regulation by pathogens (Vetukuri et al., 2012; Fahlgren et al., 2013; Åsman et al., 2016). Silencing of effector genes to avoid specific recognition and resistance has been observed for the Ps*Avr3a* locus (Qutob et al., 2013). Hence, pathogens may also show differential effector regulation depending on the host to avoid recognition. This may be of special importance for a conserved effector gene such as *PSR2.*

PSR2 may act as a master-regulator of host cell signaling.

The PSR2 protein is known to suppress phasiRNA production (Qiao et al., 2013). However, Qiao et al. (2013) demonstrated that only specific types of phasiRNA were affected. These phasiRNAs are produced via the one-hit model, which means they only require one miRNA to bind to a target as a trigger for phasiRNA production (Chen et al., 2010; Cuperus et al., 2010; Fei et al., 2013). This requires a different subset of the phasiRNA machinery than when two miRNAs are required to initiate the phasiRNA production (Adenot et al., 2006; Axtell et al., 2006; Fei et al., 2013). The machinery specific for the one-hit phasiRNA production is also responsible for the phasiRNA production from *R* genes,

which can act in *cis* and *trans* (Zhai et al., 2011); suggesting that these phasiRNAs could be affected by PSR2 expression. An up-regulation of *PSR2* in the biotrophic phase would result in a down-regulation of phasiRNA production early in infection, along with a corresponding up-regulation of *R* genes. A pathogen expressing *PSR2* would therefore release the negative regulation on *R* genes, which seems selectively disadvantageous and inconsistent with the observed virulence benefit demonstrated for this protein. However, in addition to targeting *R* genes, targets of phasiRNAs are essential for cellular processes including auxin signaling, nutrient signaling, flower and leaf development (Allen et al., 2005; Adenot et al., 2006; Hsieh et al., 2009; Zhai et al., 2015; Zheng et al., 2015). As such, downregulation of the phasiRNA production should have profound effects on development and nutrient signaling and these changes may be advantageous to the pathogen.

There may also be another advantage of PSR2, especially for broad host range pathogens: Although, hosts may differ in which genes are specifically regulated by phasiRNAs (Zheng et al., 2015), the pathogen short-circuits this form of negative regulation by targeting the biogenesis machinery, rather than specific phasiRNAs. This sudden release of multiple genes simultaneously from their negative regulation is likely to have a substantial effect on the host cell, probably to the pathogen's advantage, and may explain the widespread presence and functional conservation across many oomycetes (Xiong et al., 2014). We therefore hypothesize, that up-regulation of Pi*PSR2* in the early phase functions to massively deregulate many otherwise repressed genes in the plant cell. Genes typically under repression would include genes controlling plant functions which require rapid transcriptional responses, for instance to an unpredictable stimulus (such as pathogens), and/or genes associated with tissue-specific expression such as many developmental processes (Carlsbecker et al., 2010; Shivaprasad et al., 2012; Zhai et al., 2015). The advantages of targeting the entire population of phasiRNAs simultaneously through a master regulator likely outweigh the cost of potentially releasing some *R* genes from negative regulation.

Experimental Procedures

Plant and pathogen material.

Seeds of *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* LA0114 were surface-sterilized (3 sec 70% Ethanol; 30 sec ~5% NaOCl; three times 3 min sterile water). Seeds were put on 1.2% H_2O-agar, incubated in the dark for three days (day-night temperature cycle of 18°C /15°C [16 h/ 8 h]) and transferred to a cycle of 16 h 166 ± 17µmol quanta*m-2*s-1light and 8h dark with equal temperature conditions. Nine days post-planting (dpp) the seedlings were transferred to vessels (three to four seedlings per vessel) with 0.5% MS medium (Murashige and Skoog, 1962), containing 1% sucrose.

Thirteen isolates of *P. infestans* (NL10001, NL88069, NL90128, IPO-C, IPO428-2, 3928A, D12-2, T15-2, T20-2, T30-4, 4/91, 20/01 and 08-ANI-3) were grown on Rye-sucrose agar with 100µg/ml Ampicillin, 10µg/ml Amphotericin B and 20µg/ml Vancomycin (Caten and Jinks, 1968). The plates were incubated at 18°C in the dark, with the exception of T20-2, which was incubated at 12°C in the dark.

Cloning and sequencing of Pi*PSR2.*

P. infestans mycelium was sampled directly from the plate, shock-frozen in liquid nitrogen, ground using metal beads and DNA was extracted according to Edwards et al. (1991). Pi*PSR2* genes were amplified using 1U Platinum® *Taq* DNA polymerase High Fidelity (Thermo Scientific, USA; 0.1mM dNTPs, 2mM $MgSO_4$, 0.5µM primers (Table S3) and 100-200ng template DNA). The PCR reaction included 3min at 94°C, 35 cycles of 30sec 94°C, 30sec 58°C 3 min 68°C, and 5min 68°C. Primers were designed manually to match the flanking regions of the Pi*PSR2* open reading frame. PCR products were purified using the peqGOLD Cycle-Pure Kit (Peqlab, Germany) and cloned into a pCRTM4-TOPO® plasmid using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, USA).

Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany) or the NucleoSpin Plasmid Kit (Macherey-Nagel, Germany). Pi*PSR2* genes were sequenced at Eurofins MWG Operon (Germany). Primers to close the gaps in the sequences were designed manually (Table S3).

Quality control of Pi*PSR2* sequences.

To verify the cloned sequences, we performed a blastn search (Camacho et al., 2008) against the *P. infestans* reference genome (Haas et al., 2009) on NCBI. Only sequences which resulted in Pi*PSR2* as the best hit and showed full length coverage and an e-value of 0 were used for further analyses. Additionally, we confirmed that the cloned Pi*PSR2* sequences were predicted to encode a full length aa sequence using the "translate" function of ExPASy (Gasteiger et al., 2003) or the Sequence Manipulation Suite (Stothard, 2000). For NCBI accessions see Table S4.

Extraction of *PSR2* and reference gene sequences.

We downloaded raw sequence data of six *P. infestans* genomes (DDR7602, P13626, P13527, P17777, NL07434 and LBUS5; Yoshida et al., 2013) from NCBI (Table S5) and used Trinity v. 2.0.6 (Grabherr et al., 2011) to *de novo* assemble the genomes. We conducted blastn using BLAST v. 2.2.30+ with the Pi*PSR2* nucleotide sequence (PITG15152) from the *P. infestans* reference genome against the *de novo* assemblies. The putative Pi*PSR2* hits were aligned with the Pi*PSR2* reference sequence to identify the correct genes (File S1).

We retrieved *P. sojae* sequences from Wang et al. (2011) and *P. parasitica* sequences using blastp with the T30-4 PSR2 protein sequence on NCBI using a cutoff of a query coverage > 90%, identity > 90% and e-value of 0 (Table S5).

Reference gene sequences were extracted from the Yoshida et al. (2013) dataset, including also 06_3928A, corresponding to isolate 3928A in our lab cultures. The reference genes were Pi*ATP5D*, Pi*Elf1α* (partial), and Pi*AGO1.*

PSR2 amino acid identity.

We calculated pairwise aa identity using the "ident" function from the sequence manipulation suite. Alignments were created with MAFFT v. 7.127b (Katoh and Standley, 2013) using local pairwise alignment setting L-INS-I. *Pp*PSR2 from *P. parasitica* P10297 was excluded because the sequence was partial.

Population genetic analyses.

The *PSR2* and reference sequence datasets were aligned using the CLUSTAL-W function in MEGA v. 5.2.2 (Kamura et al., 2011; Figure S8-S11) with Ps*PSR2* as the outgroup. Basic summary statistics and tests of neutrality were conducted using DnaSP v. 5.10 (Librado and Rozas, 2009). D12-2, T15-2 and T20-2 were excluded from the analyses, because they belong to the same breeding population as T30-4 (Drenth et al., 1995).

Phylogenetic analysis.

We downloaded available mitochondrial sequences from the supplemental data of Martin et al. (2015). Sequences were aligned with MAFFT v. 7.127b using the L-INS-I settings. We built a maximum likelihood phylogeny (Tamura-Nei model, 1000 bootstraps, uniform rates and partial deletion with a cutoff of 99%) using MEGA v. 5.2.2.

Protein structure prediction.

*Pi*PSR2 secondary structure was predicted via PREDISI (Hiller et al., 2004) and PSIPRED (Jones, 1999) in the Quick2D bioinformatics toolkit (Biegert et al., 2006). The consensus structure was calculated from the average confidence value of an aa in the α-helix, considering only residues with average confidence values >5. W- and Y-motifs were identified by combining manual (using motif variants from Jiang et al. (2008) and Boutemy et al. (2011) as references) and MAST v. 4.11.2 (Bailey and Gribskov, 1998) searches; settings were: normal mode, 0-order model of sequences, searching the given strand and returning up to 20 motifs with any given number of repetitions.

Plant inoculations.

We used seedlings of *S. lycopersicum* cv. Moneymaker to compare pathogen strength of seven different *P. infestans* isolates (NL10001, NL88069, NL90128, IPO-C, D12-2, T15-2 and T20-2). Zoospores were isolated from three to four weeks old *P. infestans* mycelium by incubating the mycelium with 10ml H_2O (4°C) for at least two hours at 4°C. Spore concentrations were determined

using a Neubauer improved chamber and $5*10^4$ spores/ml were used. For NL10001 and NL90128, a spore concentration of $2*10^4$ was used, because they yielded fewer zoospores. Seedlings were inoculated at 28-30 dpp with 10µl of 4°C (i) zoospore solution or (ii) H_2O (mock control) per leaflet. All solutions were kept on ice during the procedure. We sampled three replicates at 72 hpi.

S. lycopersicum cv. Moneymaker and *S. pimpinellifolium* LA 0114 were inoculated with *P. infestans* IPO-C, D12-2 and T20-2 at 28-30 dpp as described above using $5*10^4$ spores/ml. We sampled three replicates per time point (0 hpi, 24 hpi, 48 hpi, 72 hpi and 96 hpi). *S. pimpinellifolium*-IPO-C at 96 hpi had four replicates.

Evaluation of necrotic area.

Host symptoms and *P. infestans* spread were evaluated by measuring the relative necrotic area. We assessed bleached (100% EtOH) leaflets with a SteREO Discovery V8 binocular (AxioCam ICc 5 camera; Zeiss, Germany). The area of leaflets and the necrotic lesions were measured with the ZEN lite 2012 (blue edition) software (Zeiss, Germany). To calculate the relative necrotic area we divided the total area of necrotic lesions by the total area of the leaflet. Differences of the test inoculations to the respective mock controls were calculated with a two-tailed t-test in Excel 2013. Differences within the time series datasets were evaluated using a Kruskal-Wallis test with a post-hoc Tukey test using the R CRAN package PMCMR. p-values were adjusted using a Bonferroni correction.

Trypan blue staining.

The bleached leaflets were stained with a Trypan blue–lactophenol staining (modified from Jones and Deverall, 1977). In brief: leaflets were covered with Trypan blue–lactophenol solution, cooked at 99°C until boiling, exchanged with chloral hydrate solution (0.5g/ml) and stored in fresh chloral hydrate solution. Leaflets were evaluated using an Axiophot microscope (AxioCam ICc 5 camera; Zeiss, Germany).

RNA extraction and cDNA synthesis.

RNA was extracted from each replicate and time point (pooling three to four leaflets per replicate) using the Universal RNA/miRNA Purification Kit (Roboklon, Germany). To check RNA quality, 5µl RNA were treated with 6µl deionized formamide, incubated at 65°C for 5min, followed by 5min incubation on ice and then RNA quality was evaluated on a 2% agarose gel.

cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) using 1000ng total RNA as input. The cDNA was diluted 1:1 with RNase free H_2O. We performed a –RT control to test for DNA contamination using *Solanum Elf1α* primers (Table S3). The PCR reaction (20µl) included: 1x Green GoTaq® Flexi Buffer, 2mM $MgCl_2$, 0.1mM dNTPs, 0.5µM per primer, 1U GoTaq® Polymerase (Promega, USA) and 3µl template cDNA; using the following protocol: 5 min 94°C, 35 cycles of 30sec 94°C, 30sec 56°C for and 1.30min 72°C, followed by 7 min 72°C.

Presence of *Phytophthora infestans.*

Presence of *P. infestans* in our inoculations and absence from mock controls was validated using *Phytophthora* specific *cytochrome oxidase subunit 2* (*COX2*) primers (Hudspeth et al., 2000; Table S3). The PCR reaction was performed as described as above.

Expression of biotrophic and necrotrophic marker genes of *Phytophthora infestans.*

We analyzed the *P. infestans* life cycles in the two different host species using the following marker genes: in planta *induced O* (Pi*ipiO*), *Haustorial membrane protein 1* (Pi*HMP1*), *Suppressor of necrosis 1* (Pi*SNE1*), Phytophthora infestans *necrosis* Phytophthora *protein 1* (Pi*NPP1.2*) and *Cell division cycle 14* (Pi*Cdc14*) (van West et al., 1998; Ah Fong and Judelson, 2003; Kanneganti et al., 2006; Avrova et al., 2008; Kelley et al., 2010). Pi*H2a* was used as a biomass marker gene. Primers were designed using NCBI primer blast (Ye et al., 2012). PCR reactions (20µl) consisted of 1x Green GoTaq® Flexi Buffer, 1.25mM $MgCl_2$, 0.1mM dNTPs, 0.2µM per primer, 3µl cDNA template and 2U GoTaq® Flexi DNA Polymerase (Promega, USA). The reaction conditions were as follows: 4min 94°C, several cycles of 30sec 94°C, annealing for 30sec, 1.30min 72°C, followed by 7min 72°C (primer sequences, annealing

temperatures and number of cycles in Table S3). Band intensity was evaluated using ImageJ (Abramoff et al., 2004) and relative abundance was calculated according to Zuluaga et al. (2016).

Expression analyses in *Phytophthora infestans* infections.

We performed a quantitative RT-PCR (qRT-PCR) with Pi*PSR2* with time points 24 hpi to 96 hpi. Primers were designed manually or using NCBI primer blast (Table S3). The qRT-PCR was performed in a CFX Connect™ Real-Time System (Bio Rad) using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad, USA). The settings were as follows: 3min 95°C, 45 cycles of 10sec 95°C and 30sec 60°C. Additionally, a melting curve was performed. As *P. infestans* reference genes we used Pi*H2a* and Pi*Elf1α* and as a host reference gene we used the *Translation Initiation Factor 3 subunit H* (*TIF3H*). The relative expression was evaluated according to Pfaffl (2001). Significant differences between isolates, hosts or time points were calculated using a Mann-Whitney U-test (Mann and Whitney, 1947). To estimate *P. infestans* growth *in planta*, we calculated the relative expression of Pi*H2a* and Pi*Elf1α* using *TIF3H* as the reference gene. To correlate *P. infestans* growth with Pi*PSR2* expression, we used a Spearman correlation to calculate correlation of relative Pi*H2a* and relative Pi*PSR2* expression. To test for isolate specific expression, CT-values of Pi*PSR2* and *PITG12646* of the three isolates were normalized with Pi*H2a* and Pi*Elf1α* and compared with each other. Significant differences were calculated using a Mann-Whitney U-test.

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Figures and Tables

Table 1 Population genetic analyses of Pi*PSR2* and reference genes

Gene	Dataset	No. of individuals	Analyzed region	π total * (sites) †	π_s * (sites) †	π_a * (sites) †	π(a)/π(s)	S‡ (nonsyn, syn)	Haplotype diversity	McDonald-Kreitman G-value (p-value)
PiP*SR2*	full-length	16	1-2322	2.27 (2283)	3.54 (526.9)	1.90 (1753.1)	0.535	27 (20, 7)	0.983	**4.427 (0.04)**
	N-terminus		1-186	4.84 (186)	11.47 (49.0)	2.30 (137.0)	0.200	5 (3, 2)	0.650	0.028 (0.87)
	C-terminus		187-2322	2.05 (2097)	2.81 (478.0)	1.83 (1616.0)	0.650	22 (17, 5)	0.967	**5.274 (0.02)**
PiP*SR2*	full-length	8$	1-2322	1.64 (2283)	3.73 (526.9)	1.02 (1753.1)	0.273	10 (6, 4)	0.929	0.358 (0.55)
ATP Synthase subunit δ	full-length	6$	1-510	2.00	8.83	0.00	0.000	2 (0, 2)	0.533	Nd
AGO1	full-length	8$	1-2784	0.55	1.00	0.41	0.407	4 (2, 2)	0.821	Nd
Elf1α	partial	7$	304-1173	6.68	24.60	0.87	0.035	12 (1, 11)	1.000	Nd

*all π-values times 10^{-3}, † total number of sites analyzed excluding gaps, ‡ number of segregating sites, $ isolates from the Yoshida et al. (2013) dataset, Nd not determined

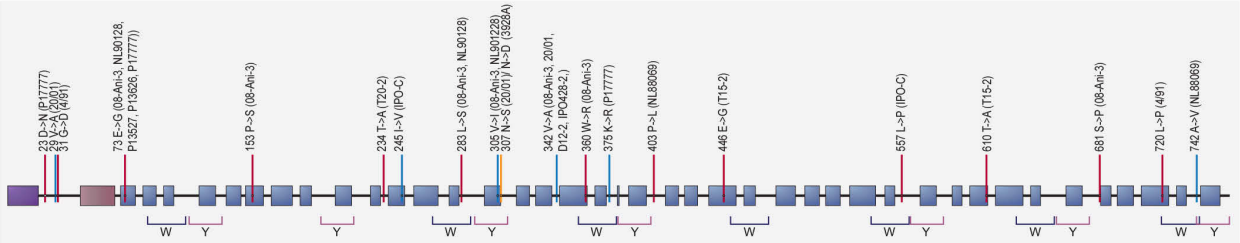
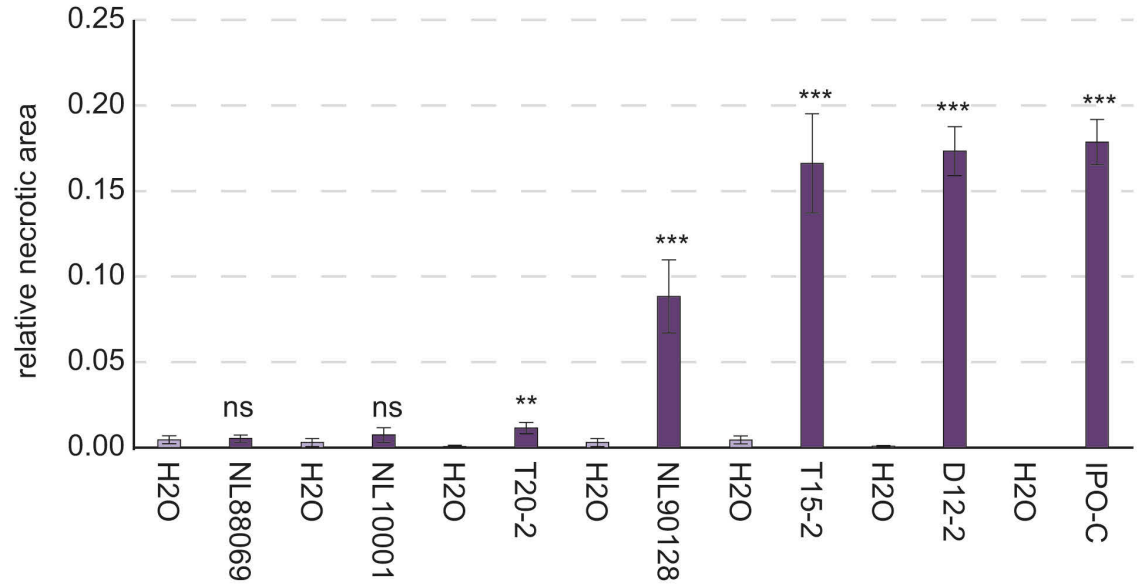


Figure 1. *Pi*PSR2 secondary structure.

The consensus sequence of *Pi*PSR2 has a predicted signal peptide (purple box) and 40 α-helices (blue boxes). The RxLR-dEER motif (light pink box) lies within aa 47 to 62. Polymorphic residues with similar chemical properties (blue lines); radical changes (red lines); isolates carrying minor allele in parentheses above residues. Predicted W- and Y-motifs are below the structure.

a



b

	Position												Symptom strength
Strain	**73**	**234**	**245**	**283**	**305**	**403**	**446**	**464**	**557**	**610**	**682**	**742**	***S. lycopersicum***
T30-4	E	T	I	L	V	P	E	L	L	T	T	A	Not determined
IPO-C	.	.	V	.	.	.	.	F	P	.	M	.	strong
D12-2	.	.	.	.	.	.	.	F	.	.	M	.	strong
T15-2	.	.	.	.	.	.	G	F	.	A	M	.	strong
NL90128	G	.	.	S	I	.	.	F	.	.	M	.	medium
T20-2	.	A	.	.	.	.	.	F	.	.	M	.	weak
NL10001	.	.	.	.	.	.	.	F	.	.	M	.	weak
NL88069	.	.	.	.	.	L	.	F	.	.	M	V	weak

Figure 2. Correlation of symptom strength with PSR2 sequence variation.

Relative necrotic area of seven *P. infestans* isolates on *S. lycopersicum* cv. Moneymaker (dark purple); mock control (light purple). Significance was determined using a two-tailed t-test (ns=not significant, *<0.05, **<0.01, ***<0.001, $n_{(leaflets)}$=33-162) (a). Polymorphic aa positions at PSR2 in the investigated isolates. A '•' indicates where the allele matches the reference allele, T30-4. Symptom strength is given to the right of the alignment (b).

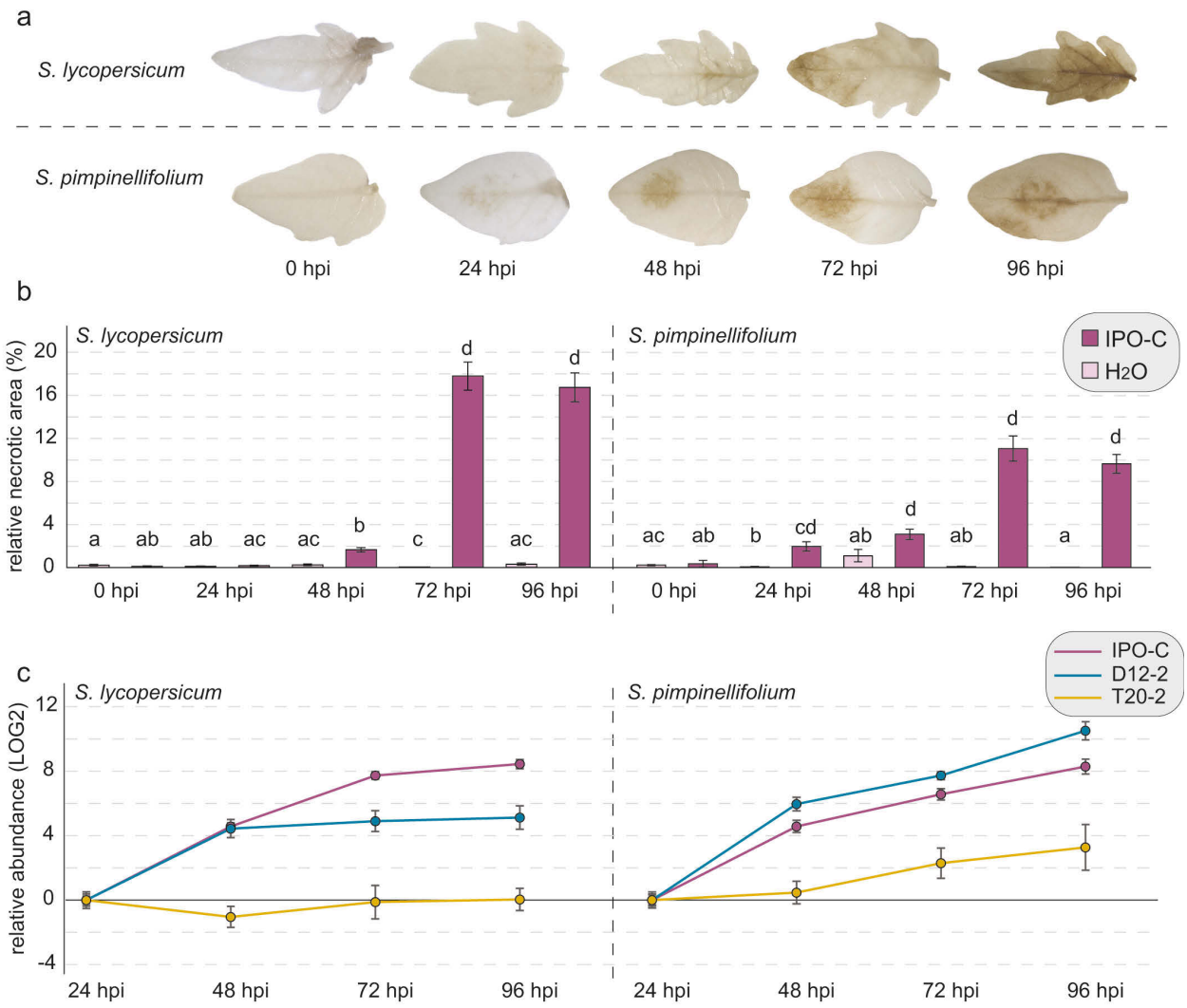


Figure 3. Progression of infection by *P. infestans* isolate IPO-C.

Representative leaflets of the infection by *P. infestans* IPO-C from 0 hpi to 96 hpi on *S. lycopersicum* and *S. pimpinellifolium* (a). Relative necrotic area of the IPO-C infection in *S. lycopersicum* and *S. pimpinellifolium* from 0 hpi to 96 hpi. Letters correspond to the different statistical groups (p-value <0.05) based on an One-way ANOVA with a Tukey post-hoc test ($n_{(leaflets)}$=56-119 (*S. lycopersicum*), $n_{(leaflets)}$=77-127 (*S. pimpinellifolium*)) within species (b). Relative abundance of *P. infestans* isolates IPO-C (purple), D12-2 (blue) and T20-2 (yellow) over time in both hosts, determined via qRT-PCR as the average relative expression of the genes Pi*H2a* and Pi*Elf1α* normalized with the plant host gene *TIF3H* (c). Error bars correspond to the SEM.

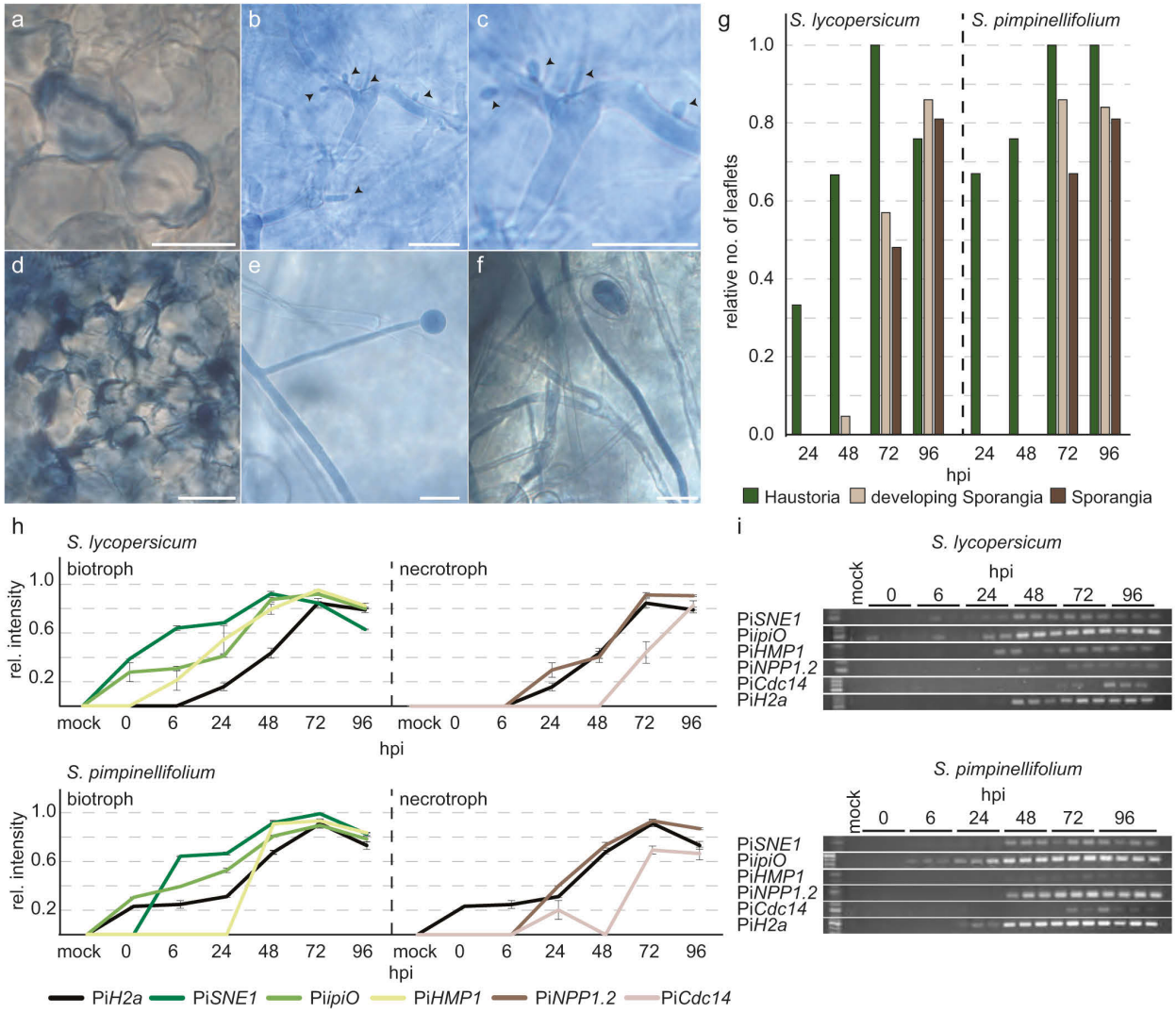


Figure 4. *P. infestans* infection and life cycle.

Trypan blue staining of leaflets with *P. infestans* show an encysted zoospore infecting *S. pimpinellifolium* cells (a), a *P. infestans* hyphae with haustoria (b, c), necrotic cells in *S. lycopersicum* (d), developing sporangia (e) and hyphae and mature sporangia (f). Scale bars=0.02 mm. The relative number of leaflets with haustoria (green), developing sporangia (beige) and mature sporangia (brown) in *S. lycopersicum* and *S. pimpinellifolium* infections by *P. infestans* IPO-C at 24 hpi to 96 hpi ($n_{(leaflets)}$=21-31) (g). Relative intensity of marker genes for the biotrophic and necrotrophic phase from 24 hpi to 96 hpi in *S. lycopersicum* and *S. pimpinellifolium* infections by *P. infestans* IPO-C. Error bars show the SEM (h).

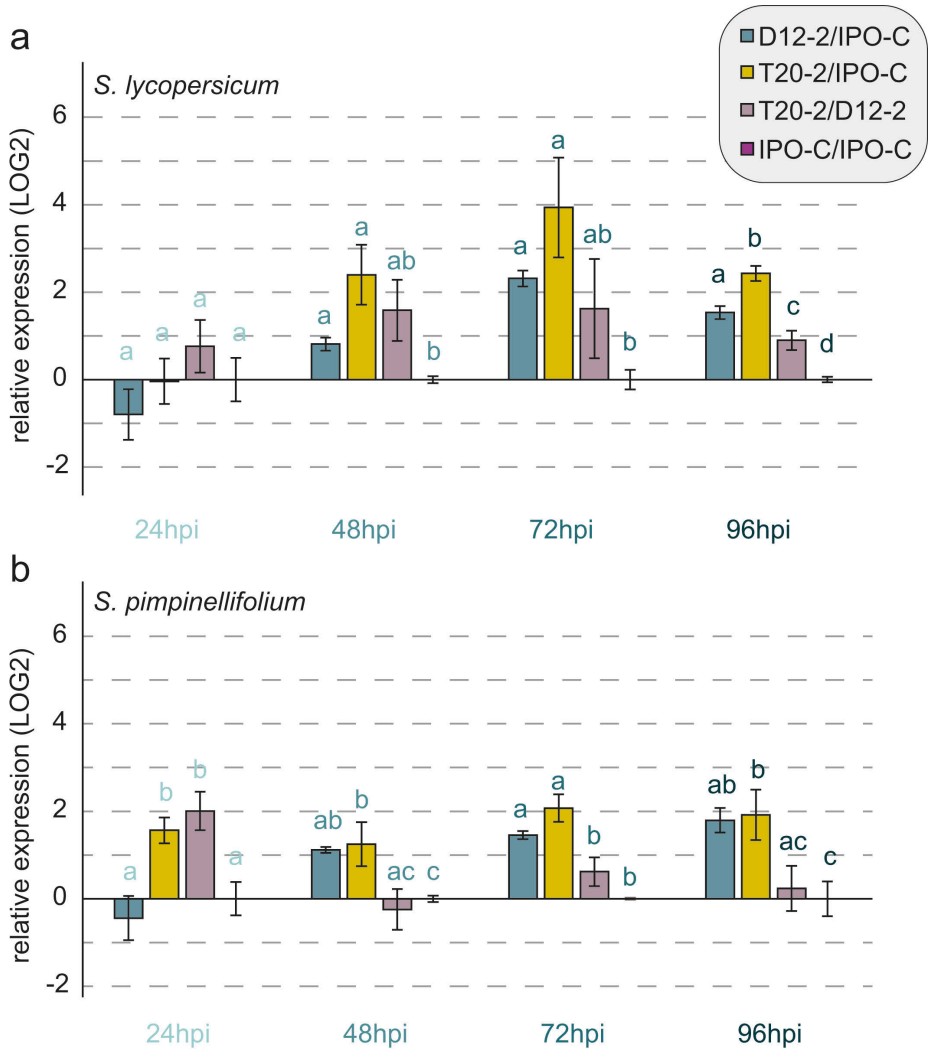


Figure 5. Expression differences of Pi*PSR2* in the different isolates.

Pairwise relative expression of Pi*PSR2* on *S. lycopersicum* (a) and *S. pimpinellifolium* (b) at 24, 48, 72 and 96 hpi. Expression was measured using qRT-PCR. For normalization, the reference genes Pi*H2a* and Pi*Elf1α* were used. Error bars indicate the SEM. Significance classes are given as letters above the bar graph. Significance was calculated within one time point.

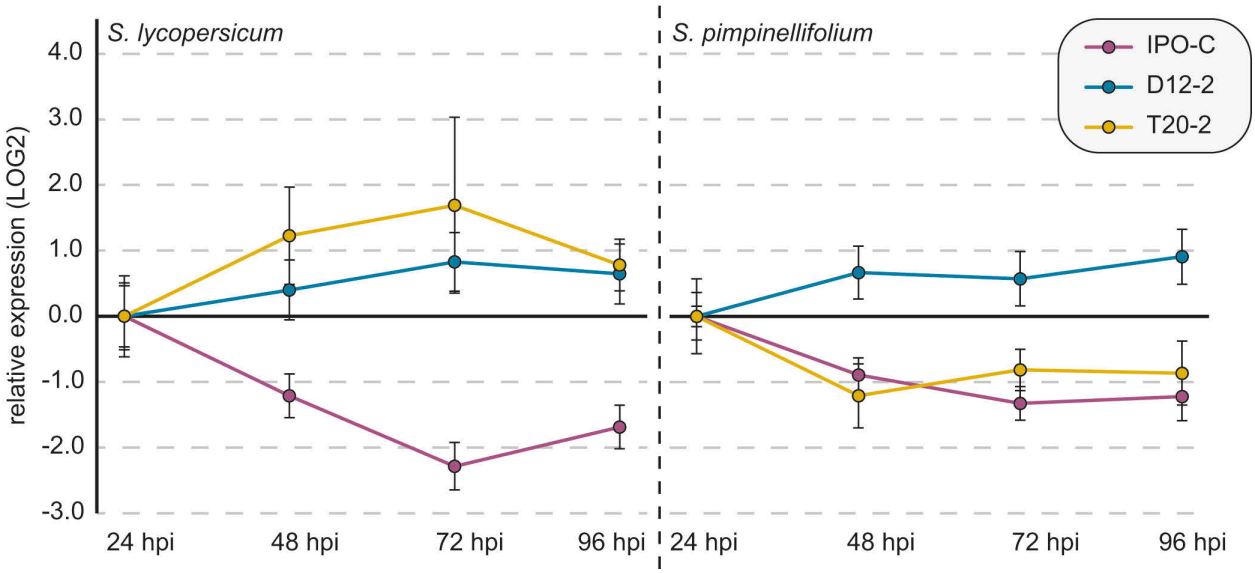


Figure 6. Relative expression of Pi*PSR2*.

Relative expression of Pi*PSR2* from IPO-C (purple), D12-2 (blue) and T20-2 (yellow) from 24 hpi to 96 hpi in *S. lycopersicum* and *S. pimpinellifolium* was determined via qRT-PCR. Pi*PSR2* expression was normalized using the reference genes Pi*H2a* and Pi*Elf1α*. Error bars indicate the SEM.

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