Expression profiling across wild and cultivated tomatoes supports the relevance of early miR482/2118 suppression for Phytophthora resistance

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1. Introduction

Resistance proteins (R-proteins) are fundamentally important in plant pathogen interactions. They recognize pathogen molecules, called effectors, which are secreted by pathogens to hijack plant immune responses [1,2]. Upon recognition, R-proteins trigger a pathogen-specific immune response [3,4]. Such immune responses include the hypersensitive response (HR), resulting in the release of reactive oxygen species, and can ultimately lead to cell death.

Misregulation of R-genes carries high fitness costs. Over-expression of R-genes in the absence of a pathogen can severely decrease fitness [5,6]. By contrast, insufficient R-gene expression during pathogen attack can allow for pathogen infection [7]. While several regulatory mechanisms are at play for different R-genes and R-proteins [8,9], negative regulation via small RNAs was proposed to globally buffer R-gene expression to avoid misregulation [10].

One example of negative regulation of R-genes, specifically of nucleotide-binding site leucine-rich repeats (NBS-LRRs), is suppression by the microRNA (miRNA) family miR482/2118 [11–13]. Targeting of miR482/2118 leads either...
to the degradation of NBS-LRR mRNA or to an inhibition of the translation of the corresponding mRNAs [14]. The family is one of the most labile miRNA families, displaying low sequence conservation, even between closely related species, despite its widespread presence in the plant kingdom [11,13,15]. Its diversity is in part a consequence of the amino acid variability of its target sequence [16].

A major pathogen of cultivated tomato (Solanum lycopersicum) is Phytophthora infestans. However, P. infestans not only infects crops but also their wild relatives [17–22]. Populations of wild tomato species, given that they are not subjected to breeding, have experienced different evolutionary histories with P. infestans compared to the cultivated tomato. In fact, wild tomatoes harbour R-genes that effectively contribute to the resistance to this pathogen [19,23].

Phytophthora infestans’s vast effector repertoire is likely the result of a constant adaptation to its diverse hosts [24]. Among P. infestans’s effectors, two were recently identified, which suppress the host’s RNA silencing machinery [25–26]. Suppression of the plant RNA silencing pathways would release miRNA targets, including NBS-LRRs, from their miRNA-mediated suppression. Therefore, it has been hypothesized that the regulation of R-genes by miR482/2118 may have evolved into a pathogen detection mechanism, i.e. a counter-defence mechanism by which pathogen-mediated RNA silencing suppression activates the plant immune system [13]. This is at odds with the observed positive influence on pathogen virulence by these effectors [25] and suggests a complex network of NBS-LRR regulation during the infection of plants by their pathogens.

In this study, we analysed how coevolution of tomatoes and their pathogen P. infestans has shaped miRNA-mediated NBS-LRR regulation and how this regulatory network contributes to resistance in tomato. We first identified miR482/2118 targets associated with P. infestans defence in S. lycopersicum. Next, we studied the expression of SlmiR482/2118 and a set of 12 NBS-LRRs in S. lycopersicum during infection by P. infestans. Although the expression of NBS-LRRs is undoubtedly regulated by multiple mechanisms in addition to negative regulation via miRNAs, we observe examples of strong coregulation between members of miR482/2118 and their targets. Combining comparative expression analyses of members of the miR482/2118 family in three closely related tomato species (S. lycopersicum, Solanum pimpinellifolium and Solanum arcanum) and analyses of host resistance led to two observations: (i) the least resistant tomato, S. lycopersicum, showed downregulation of several miRNAs from 24 to 96 hours post-inoculation (hpi) relative to the mock control, while its more resistant wild relatives did not and (ii) downregulation of miR482a and miR482f during early time-points of infection (6 hpi) correlated with resistance to P. infestans. Based on these observations, we hypothesize that global pathogen-mediated RNA silencing suppression is more effective in cultivated tomato than in its wild relatives.

2. Material and methods

(a) Plant material and Phytophthora infestans inoculation

Seeds of S. arcanum were surface sterilized using approximately 5% NaOCl (30 s), washed 3 × 3 min in sterile H2O, plated on 1.2% H2O agar and incubated in dark for 3 days (16 h/8 h with 18 C/15 C). Afterwards, the seeds were transferred to a 16 L (166 ± 17 µmol quanta m–2 s–1): 8 D regime. Nine days post sterilization (dps), seedlings were transferred to 0.5% Murashige & Skoog medium [27] with 1% sucrose.

The isolate, IPO-C, of P. infestans was grown on rye-sucrose-agar plates (with 100 µg ml–1 ampicillin, 10 µg ml–1 amphotericin B and 20 µg ml–1 vancomycin [28]) at 18 °C in the dark. Zoospores were isolated and leaflets of S. arcanum were inoculated at 28 dps as described in de Vries et al. [29]. Three biological replicates (three to four seedlings each) were sampled per treatment and time-point (0 hpi, 6 hpi, 24 hpi, 48 hpi, 72 hpi and 96 hpi).

(b) RNA extraction, miRNA purification and cDNA synthesis

Total RNA of S. arcanum was isolated using the Universal RNA/miRNA Purification Kit (Roboklon, Berlin, Germany). RNA from S. lycopersicum and S. pimpinellifolium was used from de Vries et al. [29]. miRNA was purified using the Dynabeads miRNA Purification Kit (Thermo Scientific, Massachusetts, USA).

cDNA libraries for mature miR482/2118 expression analyses were created using miScript Plant RT Kit (Qiagen, Hilden, Germany) using 250 ng total RNA and diluted 1 : 10 with nuclease-free H2O. cDNA libraries for all other expression analyses were created with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) using 1000 ng total RNA and random hexamer primers and libraries were diluted 1 : 1 with nuclease-free H2O.

cDNA libraries for the modified 5’RNA ligase-mediated rapid amplification of cDNA ends (5’RLM-RACE) were created using the GeneRacer Kit (Invitrogen, California, USA) using 50–100 ng mRNA from infections (24 and 48 hpi) and mock (48 hpi). To identify miRNA cleavage sites, the protocol was modified to omit the enzymatic digest of the cap and proceed directly to the ligation of the 5’ GeneRacer RNA oligo adapter. The SuperScript III RT Module (Invitrogen, California, USA) with the GeneRacer Oligo dT Primer was used for reverse transcription.

(c) 5’RLM-RACE

Amplification of 5’RLM-RACE products was performed (1 × High Fidelity PCR buffer, 0.6 µM GeneRacer 5’ primer, 0.2 µM of the gene specific primer (electronic supplementary material, table S1), 200 µM dNTPs, 1 mM MgSO4, 3% DMSO and 0.5U Platinum Taq DNA Polymerase High Fidelity) followed by a nested PCR, using 1 µl of the PCR product in a 50 µl reaction (1 × High Fidelity PCR buffer, 0.2 µM GeneRacer 5’ nested primer, 0.2 µM of the nested gene specific primer (electronic supplementary material, table S1), 200 µM dNTPs, 1 mM MgSO4 and 0.5U Platinum Taq DNA PolymeraseHigh Fidelity).

PCR products were amplified with a Phusion High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA) and cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, California, USA).

(d) Confirmation of infection and infection progress

To confirm successful infection and study the disease progression, leaflets of S. arcanum seedlings were analysed microscopically. The relative necrotic area and pathogen structures were determined according to [29]. Statistical differences in necrotic area over time and between mock and infections were estimated using a Kruskal–Wallis test [30] with a Tukey and Kramer post hoc test, using a Tukey distance approximation [31]. For comparisons of the relative necrotic area between
species, normal distribution of the data was evaluated using a Shapiro–Wilk test [32] and then tested for significant differences using a Mann–Whitney U test [33] in R v. 3.2.1. To determine the abundance and life cycle progression of *P. infestans* at the molecular level, expression of three biotrophic, two necrotrophic and one biomass marker gene were analysed according to [29].

(e) Identification of miR482/2118 family members

Members of miR482/2118 from *S. lycopersicum* and *S. pimpinellifolium* have been previously identified in de Vries et al. [15]. Members of miR482/2118 from *S. arcanum* were identified via a BLASTn against the *S. arcanum* genome using miR482/2118 pre-cursor sequences of *S. lycopersicum* as query. The best hits in *S. arcanum* were aligned to the SmiR482/2118 precursor sequences and the mature miR482/2118 sequences were determined. Folding of *S. arcanum* miR482/2118 precursors into hairpins was predicted using RNAfold [34] (electronic supplementary material, figure S1).

(f) Selection of *R*-genes

We chose *R*-genes that were (i) predicted to be targeted by one or more members of miR482/2118 and (ii) associated with resistance to *P. infestans*. The 52 potential miR482/2118 target genes [15] were used as queries for a BLASTn-search against the NCBI nt/nt database limited to *S. lycopersicum*. The best functional annotated BLAST hit (e.g. excluding hits to entire chromosomes) was recorded. Hits with an e-value of 0, query coverage greater than 90% and an identity greater than 85% to an *S. lycopersicum* genome using miR482/2118 pre-cursor sequences of *S. lycopersicum* were identified via a BLASTn-search against the NCBI nt/nt database limited to *S. lycopersicum*. The best functional annotated BLAST hit (e.g. excluding hits to entire chromosomes) was recorded. Hits with an e-value of 0, query coverage greater than 90% and an identity greater than 85% to an *R*-gene associated with resistance against *P. infestans* in *S. lycopersicum* or the resistance gene analogues (RGA) complex were determined as likely to be associated with resistance to *P. infestans*.

(g) qRT-PCR

qRT-PCR was performed using the miScript SYBR Green PCR (Qiagen, Hilden, Germany). miR482/2118 forward primers were designed based on the mature miR482/2118 sequences. miR482/2118 primer specificity was tested by creating a qRT-PCR product for each primer. These qRT-PCR products were purified, and each primer was tested with each qRT-PCR product to determine if and at what annealing temperatures the primers would bind to other miR482/2118 paralogues. For all miR482/2118 primers a binding-specific annealing temperature was determined (electronic supplementary material, table S1). The only exceptions were the primers for SmiR482h and SmiR482g, which annealed to miR482h as well as miR482 at all annealing temperatures. SmiR482h was specific because of its slightly different mature miRNA sequence (electronic supplementary material, table S1). As a control, the expression of mature SmiR156a/b/c, SmiR166a/b, SmiR168a/b and SmiR172a/b was determined. miR390a was used as a reference due to its constant expression across treatments and time-points according to BesKnaa v.1 [35].

Expression of NBS-LRRs in *S. lycopersicum* was determined using the SsoAdvanced Universal SYBR Green Supermix (eectronic supplementary material, table S1; Bio-Rad, California, USA). As reference genes, we used SAND [15], TIP41 [15] and Translation Initiation Factor 3 subunit H (TIF3H; [29]).

Relative abundance and progression of *P. infestans* were measured using *Histone2a* (*PH2a*). Expression of *PH2a* at time-points 24 to 96 hpi was set relative to its expression at 24 hpi. The data were normalized with the plant reference genes (SAND, TIP41 and TIF3H).

Relative expression was calculated according to [36]. Data were tested for normality using a Shapiro–Wilk test [32] and equal variance using R v. 3.2.1. Comparisons between infections and mock control were tested using either a two-sample t-test or a Welch two-sample t-test for normally distributed data or a Mann–Whitney U-test [33] for non-normally distributed data.

3. Results and discussion

(a) One-third of potential nucleotide-binding site leucine-rich repeats targets have high identity to *Phytophthora infestans*-associated resistance genes

We screened for NBS-LRRs that are potential targets of miR482/2118 and classified as *R*-genes for *P. infestans* (electronic supplementary material, table S2). Of the 52 predicted NBS-LRR targets [15], we identified 20 which were annotated as a *P. infestans*-associated *R*-gene or the RGA complex, members of which are associated with resistance to the pathogen [37–38]. Of these 20, 17 matched a *P. infestans*-associated *R*-gene with an e-value of 0, a query coverage of greater than 90% and an identity of greater than 85% (electronic supplementary material, table S2). Therefore, approximately 33% of the predicted direct NBS-LRR targets of miR482/2118 are associated with resistance to *P. infestans*.

(b) Nucleotide-binding site leucine-rich repeats are targeted by miR482/2118 in *Solanum lycopersicum* during infection by *Phytophthora infestans* infection

Previous studies have used 5’RLM-RACE to test whether the expression of NBS-LRRs is regulated by members of the miR482/2118 gene family [13–14]. Targeting by SmiR482f of Solyc08g075630.2.1 and Solyc08g076000.2.1, which are associated with *P. infestans* defence responses (electronic supplementary material, table S2), was only shown in overexpression lines of *Nicotiana benthamiana* [14]. To test whether these NBS-LRRs are targeted by miR482/2118 in *S. lycopersicum*, we created 5’RLM-RACE libraries from *S. lycopersicum* infected with the pathogen and mock-treated (figure 1). In addition, we tested Solyc02g036270.2.1, because it is a functional miR482/2118 target [13] that is not associated with *P. infestans* resistance (figure 1; electronic supplementary material, table S2).

A cleavage site is determined by an enrichment of a specific degradation product in the 5’RLM-RACE library. This is established by cloning the degradation products of the gene of interest from the library and analysing how often a specific degradation product was cloned. If the gene of interest has a mRNA cleavage site, the majority of the clones should contain a product cut at the predicted cleavage site. All three tested genes revealed a cleavage site in the region complementary to the miR482/2118 sequences. Moreover, these cleavage products were observed in both mock-treated and *P. infestans* infected leaflets of *S. lycopersicum*. Based on clone analyses of the 5’RLM-RACE library of Solyc02g036270.2.1, 15 out of 18 clones were cleaved between nucleotide positions 11 and 12 of the mRNA binding site (figure 1a). For Solyc08g075630.2.1, all clones (24/24) were cleaved between nucleotide positions 12 and 13 of the mRNA binding site (figure 1b). For Solyc08g076000.2.1, 13/17 clones had a cleavage site between nucleotide positions 12 and 13 of the mRNA binding region (figure 1c). Some alternative cleavage products were observed for Solyc02g036270.2.1 and Solyc08g076000.2.1 (figure 1). This is in agreement with Ouyang et al. [14], who also observed an alternative cleavage site for Solyc08g076000.2.1. In summary, we demonstrate that targeting of NBS-LRRs by miR482/2118 is effective in pathogen-challenged and unchallenged plants.
(c) Co-regulation of members of miR482/2118 and their nucleotide-binding site leucine-rich repeats targets is time-dependent

Given that a third of the miR482/2118 potential targets in *S. lycopersicum* are associated with disease resistance to *P. infestans* in *S. lycopersicum*, we chose a subset of 11 NBS-LRRs associated with *P. infestans* resistance and Solyc02g036270.2.1 (as a positive control for cleavage, but a negative control in terms of *P. infestans* resistance) to study the co-regulation of NBS-LRRs and miR482/2118 in this interaction. We quantified the expression of the seven members of miR482/2118 and 12 NBS-LRRs in infected and uninfected plants across five time-points (6 to 96 hpi) (figures 2a and 3).

To identify to what degree the miRNAs show similar expression patterns in response to infection, we compared the expression of the individual miRNAs and recorded how often two miRNAs showed the same expression pattern in parallel at a given time-point, to see whether both show (i) significant upregulation, (ii) significant downregulation or (iii) no differential regulation between infection versus mock. Overall, all SlmiR482/2118 miRNAs show similar dynamics in expression, with the same expression pattern of two miRNAs for 3.1 ± 0.9 time-points, on average (figure 2a).

By contrast, two NBS-LRRs show, on average, the same expression pattern at 2.0 ± 1.3 time-points (figure 3). This is a significantly lower co-regulation compared to that observed for miR482/2118 (*p*-value = 0.0002). Such differences in co-regulation suggest that despite active targeting by miR482/2118 in *S. lycopersicum*, NBS-LRRs are likely to be regulated by other mechanisms in addition to the regulation by miR482/2118.

Next, we evaluated how often pairs of miR482/2118 and NBS-LRRs are co-regulated and what type of co-regulation they are subjected to (i.e. negative co-regulation, positive co-regulation or no differential regulation of both miRNA and target). In total (over all time-points), we evaluated 95 miR482/2118–NBS-LRR combinations (electronic supplementary material, figure S2). In 45 pairs, the NBS-LRRs are predicted to be post-transcriptionally regulated, while 50 are predicted to be translationally regulated (electronic supplementary material, table S2). If a target is post-transcriptionally regulated, one would predict a negative co-regulation of target and miRNA. This means that if the miRNA is significantly upregulated, the target should be significantly downregulated and vice versa. Nevertheless, positive correlations between miRNA and target mRNA levels have been reported [39–41]. Additionally, positive co-regulation has been observed for miRNAs [39] that suppress their targets translationally [42], suggesting that translational repression can lead to positive co-regulation. If the miRNA is not differentially regulated between infection versus mock treatment, the target should not be either.

We observed that the direction of co-regulation is not static for every miR482/2118–NBS-LRR combination but can shift between time-points. Such rapid shifts in co-regulation may result from switches between translational and post-transcriptional suppression. For example, Solyc08g076000.2.1 shows an alternating pattern of co-regulation with SlmiR482f.
We determined at which time-points co-regulation was most prevalent, suggesting a potential influence of miR482/2118 on NBS-LRR-regulation. The greatest co-regulation occurred at 48 hpi with 10/12 NBS-LRRs showing co-regulation with at least one of their respective SlmiR482/2118 members (electronic supplementary material, figure S2). High co-regulation was also detected at 6 and 72 hpi for 9/12 NBS-LRRs. All three time-points are biologically interesting: 6 hpi is a crucial time-point for infection success, as early HR significantly contributes to resistance against P. infestans [20]. Between 48 and 72 hpi, P. infestans switches from a biotrophic (i.e. requiring nutrients from a living host) to a necrotrophic phase (i.e. inducing host cell death) [29].

**Figure 2.** Expression of miR482/2118 family members in *S. lycopersicum, S. pimpinellifolium* and *S. arcanum*. Relative expression (log2) in infected compared with mock-control plants of *S. lycopersicum* (a), *S. pimpinellifolium* (b) and *S. arcanum* (c) of the seven miR482/2118 family members at 6, 24, 48, 72 and 96 hpi relative to mock control. The bars represent the average relative expression of the mature miRNAs and the error bars indicate the standard error of the mean (SEM). Significant differences of the relative expression of the miRNA in infected versus mock-treated plants at a specific time-point are indicated by *(p-value < 0.05), ***(p-value < 0.01), ****(p-value < 0.001) and ns (not significant).

*(d) Solanum arcanum is less susceptible to Phytophthora infestans than its two relatives*

We found that co-regulation of miR482/2118 with their targets was time-dependent, and more prevalent at time-points critical for infection success and transitions in the pathogen’s life cycle. To place this in context with resistance, we compared the response of three tomato species, *S. lycopersicum, S. pimpinellifolium* and *S. arcanum*, to *P. infestans*. These host species differ in their evolutionary and ecological histories.
Figure 3. Expression and co-regulation of Smlr482/2118 and their NBS-LRR targets. Relative expression (log2) of potential NBS-LRR targets of miR482/2118 in infected compared with mock-control plants of S. lycopersicum. Bars show the mean expression and error bars indicate the SEM. Statistical differences in relative expression in infected versus mock-treated plants at a specific time-point are indicated by * (p-value < 0.05), **(p-value < 0.01), *** (p-value < 0.001) and ns (not significant). Filled circles below each gene corresponds to the miRNA(s) predicted to target each NBS-LRR. Arrow heads indicate significant up or down-regulation of the members of Smlr482/2118 at a given time-point: upward arrow heads indicate significant upregulation and downward arrow heads indicate significant downregulation of the miRNA. The arrow heads are coloured according to their respective miRNA. Vertical lines between miRNA arrow heads and the relative expression of the NBS-LRR highlight significant negative co-regulation between members of the Smlr482/2118 family and their targets at a specific time-point.

*S. lycopersicum* has long been subjected to artificial selection. Furthermore, high-density monocultures of crop species can allow for higher pathogen loads and potentially higher pathogen diversity in the cultivated species [43].

*Solanum pimpinellifolium* and *S. arcanum* have partially overlapping ranges: *S. pimpinellifolium*’s habitat spans from Central Ecuador to Chile, while *S. arcanum* occurs in Northern Peru [44]. Furthermore, their habitats overlap with that of *P. infestans* [45–47], allowing for exposure to and coevolution with the pathogen. Indeed, *R*-genes associated with resistance to *P. infestans* have been isolated from *S. pimpinellifolium* [23,48]. In addition, *S. pimpinellifolium* is facultative self-compatible and *S. arcanum* is predominantly self-incompatible [44]. Mating system differences can influence the evolutionary history of the hosts and their adaptation potential. We therefore hypothesize that the different hosts will show variation in their resistance to *P. infestans* because they experienced different evolutionary histories.

In our previous study [29], we evaluated the relationship between pathogen abundance, the presence of pathogen infection structures and disease symptoms in *S. lycopersicum* and *S. pimpinellifolium*. Here, we describe our new results on *S. arcanum* and compare these with the results from *S. lycopersicum* and *S. pimpinellifolium*. The relative necrotic area of *S. arcanum* increased significantly at 48 hpi (figure 4c; electronic supplementary material, S3d,e), suggesting that the transition to necrotrrophy occurred between 48, 72 hpi. However, the number of all infection structures was lower in *S. arcanum* compared with the other two species (figure 4e). As less virulent isolates of *P. infestans* also show a reduction in haustoria compared with more virulent isolates [49], this suggests that *P. infestans* is less infective and has a delayed life cycle transition on *S. arcanum*.

Across all species, sporangia develop the earliest (48 hpi) in *S. lycopersicum* (figure 4e). The relative necrotic area 72 and 96 hpi is also the highest in *S. lycopersicum* (figure 4a–d). Taken together, this suggests that, although all species are susceptible to *P. infestans*, they are so by a variable degree: *S. lycopersicum* is likely the most susceptible, followed by *S. pimpinellifolium* and finally *S. arcanum*, which is the least susceptible of all three species.

(e) MiR482a and miR482f are candidate miRNAs for defence responses against *Phytophthora infestans*

We evaluated the miRNA expression between the tomatoes in relation to their resistance phenotype. Compared with *S. lycopersicum*, expression between pairs of miRNAs was significantly less correlated in the wild tomatoes: in *S. pimpinellifolium* pairs of miR482/2118 members showed the same expression pattern at 2.2 ± 1.2 time-points (p-value = 0.012; figure 2b) and in *S. arcanum* at 2.2 ± 0.9 time-points (p-value = 0.005; figure 2c). Lower co-regulation suggests additional gene-specific regulatory mechanisms in the wild tomatoes. By contrast, the cultivated tomato appears to have a more global co-regulation of miR482/2118 expression. These differences in co-regulation between wild and cultivated tomatoes could result from (i) differences in the evolutionary history of these plants (i.e. artificial versus natural selection) that brought about a more streamlined regulation of expression of miR482/2118 in *S. lycopersicum* or (ii) greater sensitivity to pathogen manipulation of host RNA silencing in *S. lycopersicum*, for agreement with this, most marker genes for biotrophy are expressed throughout the infection, but the sporulation marker Cdc14 was only expressed from 72 hpi onwards (electronic supplementary material, figure S3d,e), suggesting that the transition to necrophytroph occurred between 48, 72 hpi. However, the number of all infection structures was lower in *S. arcanum* compared with the other two species (figure 4e). As less virulent isolates of *P. infestans* also show a reduction in haustoria compared with more virulent isolates [49], this suggests that *P. infestans* is less infective and has a delayed life cycle transition on *S. arcanum*.
and mature sporangia of *P. infestans* were counted per time-point and are indicated by different letters above the boxes. The latter is of interest because two RNA silencing suppressors have been previously described in *S. arcanum*. Infection progress in *S. arcanum* (blue), *S. pimpinellifolium* (yellow) and *S. lycopersicum* (purple) (d). Statistical differences in relative necrotic area for the three species were calculated per time-point and are indicated by different letters above the boxes. The p-value cut-off was 0.05. Comparison of the number of haustoria, developing and mature sporangia of *P. infestans* after infection of *S. arcanum* (blue), *S. pimpinellifolium* (yellow) and *S. lycopersicum* (purple) (e). All data for *S. pimpinellifolium* and *S. lycopersicum* were published previously in de Vries et al. [29].

Next, we examined the relationship between the expression of *miR482/2118* miRNAs and the life cycle of *P. infestans*. We focused on 6, 48 and 72 hpi, because they are critical time-points during infection by *P. infestans* and they correspond to the pathogen, because the predicted NBS-LRR, because it was so far not reported to be associated with resistance to *P. infestans*.

We compared the expression patterns of *SlmiR482/2118* with those in the close relatives of *S. lycopersicum*. In *S. pimpinellifolium*, only two *SpmiR482/2118* members were significantly upregulated at 6 hpi (figure 2b). In *S. arcanum*, none of the seven *SamiR482/2118* members were upregulated at this time-point (figure 2c). Moreover, four out of seven *SamiR482/2118* were significantly downregulated at 6 hpi in *S. arcanum* (figure 2c), which was the most resistant tomato species. All of these members of *miR482/2118* have targets associated with *P. infestans* defence in *S. lycopersicum* (electronic supplementary material, table S2). In fact, the *R*-gene targets of *SlmiR482a* and *SlmiR482f* were significantly downregulated at 6 hpi in *S. lycopersicum* (figure 3). Therefore, the downregulation of *SamiR482a* and *SamiR482f* upon infection in *S. arcanum* might be related to the enhanced resistance observed in this species. This downregulation of *SamiR482/2118* in *S. arcanum* in the presence of the pathogen could allow for an earlier response to the pathogen, because the predicted NBS-LRR targets would not be repressed during the first 6 h, as they are in *S. lycopersicum*. Taken together, these results point to *miR482a* as a reference NBS-LRR, because it was so far not reported to be associated with resistance to *P. infestans*.
and miR482f as potential regulators of *P. infestans*-associated defence responses.

Given the substantial co-regulation of miRNAs and their targets at 48 and 72 hpi, we evaluated the association of miR482/2118 expression with the life cycle progression of *P. infestans* on its hosts. In the biotrophic phase (prior to 72 hpi), *P. infestans* requires a living host. High R-protein activity during this time frame could lead to earlier pathogen perception and activation of HR/cell death, which in turn would limit pathogen spread [20]. In the necrotrophic phase, *P. infestans* induces host cell death [52–53]. High R-protein activity at this time-point may not be beneficial to the host, but instead benefit the pathogen. An effective plant resistance response during the necrotrophic phase may include the suppression of cell death-inducing proteins, such as R-proteins, perhaps through an upregulation of miR482/2118. By contrast, if pathogen-mediated RNA silencing suppression was effective at these later time-points, one would expect a downregulation of miRNAs, including miR482/2118.

At 48 hpi, four SlmiR482/2118 (SlmiR482f, SlmiR482, SlmiR482/h and SlmiR5300) were downregulated specifically in infected plants (figure 2a). While this does not exclude a plant-mediated downregulation of miR482/2118, the downregulation of the non-NBS-LRR regulating miRNAs (electronic supplementary material, figure S4), indicates that pathogen-mediated RNA silencing suppression may play a role here. In agreement with this, a *P. infestans* RNA silencing suppressor, potentially involved in silencing the miRNA-mediated silencing pathway, has its highest expression in the main biotrophic phase [26]. In *S. arcanum*, three SmiR482/2118 members (SmiR482a, SmiR482b and SmiR482h) were downregulated during the infection compared to the control (figure 2c). By contrast, none were downregulated in *S. pimpinellifolium* (figure 2b).

After the transition to necrotrophy at 72 hpi, the following miRNAs were upregulated: SlmiR482a, SlmiR482b and SlmiR482f in *S. lycopersicum*, SmiR482a, SmiR482h and SmiR300 in *S. arcanum* and SmiR482a in *S. pimpinellifolium* (figure 2). None of the control SmiRNAs were significantly upregulated (electronic supplementary material, figure S4), suggesting a miRNA-specific plant response at this time-point. miR482a is upregulated at 72 hpi in the infections across all three species, despite the small lag in *S. arcanum* for the transition from biotrophy to necrotrophy. This would suggest that upregulation of miR482a is a consistent phenotype associated with a plant defence response during the necrotrophic phase of *P. infestans*. This is further supported by the negative co-regulation of SlmiR482a and its target *Solyc11g06530.1.1* at this time-point (figure 3; electronic supplementary material, S2).

### 4. Conclusion

In this study, we investigated the expression of miR482/2118 during the infection of *P. infestans* on three different tomato species. We found that co-regulation of mature SlmiR482/2118 and their targets in cultivated tomato was highest during the initial phase of infection and during the life cycle transition of *P. infestans* from biotrophy to necrotrophy. Across-species comparisons of the gene expression of mature miR482/2118 and of the strength of resistance led to two main conclusions: (i) Co-evolution of *P. infestans* and *S. lycopersicum* may have resulted in a more efficient pathogen-mediated RNA silencing suppression compared with its more resistant sister species; and (ii) miR482a and miR482f could be identified as candidate miRNAs for mediating the resistance response of tomatoes to *P. infestans*.

### Data accessibility

The precursor sequences of the *SmiR482/2118* family, have been identified in the draft genome from *S. arcanum* based on a BLASTn approach. The sequence data are made available in the electronic supplementary material.

### Author's contributions

S.d.V. and L.E.R. designed the study and drafted the manuscript. A.K. and T.K. contributed the bioinformatics analyses. S.d.V., A.K., J.K.v.D. and A.S. generated the molecular laboratory data. S.d.V., A.K. and J.K.v.D. analysed the data. All authors read and approved the manuscript.

### Competing interests

We have no competing interests.

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### References

1. Whisson SC et al. 2007 A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450, 115–118. (doi:10.1038/nature06203)
2. Fabro G et al. 2011 Multiple candidate effectors from the oomycete pathogen *Hyaloperonospora arabidopsidis* suppress host plant immunity. *PLoS Pathog.* 7, e1002348. (doi:10.1371/journal.ppat.1002348)
3. Allen RL et al. 2004 Host – parasite coevolutionary forces. *Proc. R. Soc. B* 271, 115–118. (doi:10.1098/rspb.2003.2560)
4. Stokes TL, Kunkel BN, Richards EJ. 2002 Epigenetic variation in *Arabidopsis* is regulated at the transcript level by multiple factors. *Proc. Natl Acad. Sci. U. S. A.* 99, 14570–14575. (doi:10.1073/pnas.252376699)
5. Lai Y, Eulgem T. In press. Transcript-level expression control of plant NLR genes. *Curr. Opin. Immunol.* 32, 114–121. (doi:10.1016/j.coi.2015.01.014)
6. Li X, Kapos P, Zhang Y. 2015 NRIs in plants. *Curr. Opin. Immunol.* 32, 114–121. (doi:10.1016/j.coi.2015.01.014)
7. Li Y, Yang S, Yang H, Hua J. 2007 The TIR-NB-LRR gene *SNC1* is regulated at the transcript level by multiple factors. *Mol. Plant Microbe Interact.* 20, 1449–1456. (doi:10.1094/MPMI-20-11-1449)
8. Krasileva KV, Dahlbeck D, Staskawicz BJ. 2010 Activation of an Arabidopsis resistance protein is specified by the * planta* association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22, 2444–2458. (doi:10.1105/tpc.110.073538)
9. Allen RL et al. 2004 Host – parasite coevolutionary forces. *Proc. R. Soc. B* 271, 115–118. (doi:10.1098/rspb.2003.2560)
10. Fei Q, Xia R, Meyers BC. 2013 Phased, secondary, small interfering RNAs in posttranscriptional gene silencing. *Nature* 493, 407–411. (doi:10.1038/nature11903)
11. Zhai J et al. 2011 MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs.

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21. Fooland MR, Sullenberger MT, Ashrafi H. 2015
20. Vleeshouwers VGAA, van Dooijeweert W, Govers F, 
18. Smart CD, Tanksley SD, Mayton H, Fry W. 2007
17. Garry G, Forbes GA, Salas A, Santa Cruz M, Perez 
13. Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos 
14. Ouyang S, Park G, Atamian HS, Han CS, Stajich JE, 

Plant Dis. correspondence to field and greenhouse screenings. Detached-leaflet evaluation of tomato
853 – 864. (doi:10.1007/s00425-005-0690)

Phytophthora infestans from cultivated potato and wild
solanaceous hosts in Peru. Plant Pathol. 54, 740 –

718 – 722. (doi:10.1093/pdis/doi:0150-010-

Vleeshouwers VGAA, van Dooijeweert W, Govers F, 
Kamoun S, Colon LT. 2000 The hypersensitive
response to Phytophthora infestans in Lycopersicon 
pennellii. Plant Dis. 91, 427 – 438. (doi:10.1094/
PDS-91-8-1045)

Li J et al. 2011 Identification and mapping of quantitative resistance to late blight (Phytophthora 
infestans) in Solanum habrochaites LA1777. 
Euphytica 179, 427 – 438. (doi:10.1007/s10681-010-

Fooland MR, Sullenberger MT, Ashrafi H. 2015
Detached-leaflet evaluation of tomato 
germplasm for late blight resistance and its 
correspondence to field and greenhouse screenings. 
Plant Dis. 99, 718 – 722. (doi:10.1094/PDIS-08-14-

Michalska AM, Sobkowiak S, Flis B, Zimnoch-
Guzowska E. 2016 Virulence and aggressiveness of 
Phytophthora infestans isolates collected in Poland 
from potato and tomato plants identified no strong 
specificity. Eur. J. Plant Pathol. 144, 325 – 336. 
(doi:10.1007/s10658-015-0769-6)

Zhang C et al. 2014 The Ph-3 gene from Solanum 
pimpinellifolium encodes CC-NBS-LRR protein 
conferring resistance to Phytophthora infestans.
51. Luan Y, Cui J, Li J, Jiang N, Liu P, Meng J. 2017 Effective enhancement of resistance to Phytophthora infestans by overexpression of miR172a and b in Solanum lycopersicum. *Planta* **247**, 127 – 138. Early Access Online Version. (doi:10.1007/s00425-017-2773-x)

52. Kanneganti TD, Huitema E, Cakir C, Kamoun S. 2006 Synergistic interactions of the plant cell death pathways induced by Phytophthora infestans Nep- like protein PiNPP1.1 and INF1 elicitin. *Mol. Plant Microbe Interact.* **19**, 854 – 863. (doi:10.1094/MPMI-19-0854)

53. Kelley BS, Lee SJ, Damasceno CM, Chakravarthy S, Kim BD, Martin GB, Rose JK. 2010 A secreted effector protein (SNE1) from Phytophthora infestans is broadly acting suppressor of programmed cell death. *Plant J.* **62**, 357 – 366. (doi:10.1111/j.1365-313X.2010.04160.x)