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Accumulation of miRNA and mRNA Targets in Potato Leaves Displaying Temperature-Dependent Responses to Potato Virus Y

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Abstract

We demonstrate temporal-, spatial- and temperature-dependent expression of five host microRNAs and target mRNAs in potato leaves after potato virus Y (PVY) inoculation. A novel gene, *Ny-DG*, conferring resistance to PVY was mapped on chromosome IX in the diploid potato clone DG 81-68. At 20 °C, the PVY^{NTN} strain was localized in the inoculated leaves without symptoms. At 28 °C, necrotic lesions were observed in both the inoculated leaves and the non-inoculated upper leaves and the virus spread systemically, which indicates overcoming the resistance. At 0.5 h post-inoculation and 3-day post-inoculation (dpi), PVY led to significant changes in the levels of the tested miRNAs and their targets only in a small number of cases. At 6 dpi, in the inoculated leaves at 28 °C, the increased expression of stu-miR162, stu-miR168a and stu-miR482 (PotatoMir1005353123_x15854) promoted the downregulation of their targets *DCL1*, *AGO1-2* and *Cc-nbs-lrr*, respectively. The expression of stu-miR482 (PotatoMir1005658171_x16170) and stu-miR172e remained unchanged, whereas their targets *Gpa2* and *TOE3* were downregulated. However, in the inoculated leaves at 20 °C, all five tested miRNAs and their targets showed parallel downregulation. In the non-inoculated upper leaves, changes in gene expression levels, namely downregulation, were only detected for the tested five miRNAs at 28 °C. We conclude that the expression patterns of the tested miRNAs and targets were altered differently not only at different time points post-inoculation but also in the inoculated and upper leaves, and their accumulation levels were related to the type of reaction, which in turn was dependent on temperature.

Keywords Diploid potato · Inoculated leaves · microRNA · Non-inoculated upper leaves · *Potato virus Y* · Temperature-dependent response

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Introduction

The hypersensitive response (HR) and extreme resistance (ER) are the most common defence mechanisms against viral infections in plants. HR-mediated resistance is a programmed cell death response, which leads to effective pathogen restriction in the infected cells or cells adjacent to them and is associated with necrotic lesion generation at the infection sites. ER fails in virus replication at the individual cell level and plants remain symptomless (Kang et al. 2005; de Ronde et al. 2014). The phenomenon of cells dying can also be observed in compatible plant-pathogen interactions and is known as necrosis (Hinrichs-Berger et al. 1999). HR is often temperature-dependent (Zhu et al. 2010). In the leaves of Xanthi-nc tobacco (*Nicotiana tabacum* L.) of plants possessing the *N* gene, HR was induced after tobacco mosaic virus (TMV) infection at 20 °C. At 28 °C, the resistance was overcome and tissue necrosis did not develop (Király et al. 2008). The HR-type resistance elicited in the leaves of *Nicotiana* species by oat dwarf virus (ODV) was suppressed at temperatures above 30 °C (Qian et al. 2016). The HR to the potato virus Y (PVY) strain PVY^{N} in *Solanum sparsipilum* and *Solanum sucrense* as well as the HR against the strain PVY^{O} in the potato (*Solanum tuberosum* L.) cultivar Pito was efficiently expressed at temperatures from 6 to18 °C as indicated by the development of necrosis at the infection sites and/or systemically (Valkonen 1997). However, at higher temperatures (19/24 °C), only leaf drop and mosaic symptoms developed (Valkonen 1997). The temperature-dependent HR to PVY^{O} was also observed in potato cv. Exploits (Nie et al. 2015). The mechanism by which temperature influences the outcome of HR remains to be elucidated (Valkonen 2015).

We previously reported the first temperature-dependent hypersensitivity gene, *Ny-1*, in potato cv. Rywal. This gene confers HR to both PVY^{O} and PVY^{N} strains of PVY at 20 °C; however, the resistance was overcome at 28 °C and the plants were systemically infected but no symptoms were observed (Szajko et al. 2008). In another study from our group, a temperature-independent but strain type–dependent HR was demonstrated in potato cv. Etola, whereas HR resistance to PVY^{NTN} isolate PVY-3202 and necrotic reaction to PVY^{Z}-NTN isolate PVY-3303 and $PVY^{N\text{-}Wi}$ isolate PVY-3411 were observed at temperatures ranging from 20 to 28 °C (Yin et al. 2017). Recently, in a preliminary study from our group, we found a different type of temperature-dependent potato-PVY interaction in a diploid potato clone DG 81-68. At the phenotype level, in contrast to the cv. Rywal, it exhibits a symptomless resistance to PVY^{NTN} at 20 °C and overcomes the resistance and development of necrotic lesions in both the inoculated leaves and the non-inoculated upper leaves at 28 °C. At the genotype level, DG 81-68 represents the temperature-dependent resistance to PVY, which we have found in other potato cultivars besides cv. Rywal, for example, the genes *Ny-1A* and *Ny-1S* in cvs. Albatros and Sekwana, respectively (Szajko et al. 2014). In our study, we suggested the putative resistance gene might be carried by DG 81-68 as *Ny-DG*. Given its novel reaction type towards PVY, we have chosen this diploid potato clone DG 81-68 for further research, for example, in respect to miRNA expression study.

Plant miRNAs are small endogenous non-coding RNAs that post-transcriptionally regulate gene expression by targeting specific mRNA for cleavage or translational inhibition (Bartel 2004; Voinnet 2009); they play essential roles in plant development and responses to biotic and abiotic stresses (Ruiz-Ferrer and Voinnet 2009; Khraiwesh

et al. 2012; Ramesh et al. 2014; Yin et al. 2014). Certain miRNAs are involved in antiviral immunity by regulating resistance (*R*) genes that encode proteins containing nucleotide binding (NB) and leucine-rich repeat (LRR) domains (Li et al. 2012; Shivaprasad et al. 2012; Permar et al. 2014; Chen et al. 2016a, b). Previously, we showed that a set of potato miRNAs and targets was altered in cv. Etola plants displaying partial HR and severe symptoms after infection with the $PVY^{N\text{-}Wi}$ isolate but not in plants presenting HR resistance to the PVY^{NTN} isolate or in plants displaying partial HR and necrosis to PVY^{Z}-NTN (Yin et al. 2017). Later on, these three PVY isolates were used to inoculate tobacco (cv. Samsun) plants and to study miRNA expression in PVY-tobacco interaction (Yin et al. 2019). The abundance of the majority of tested tobacco miRNAs and targets was increased upon infection by PVY^{NTN} and $PVY^{N\text{-}Wi}$ isolates, which induced severe symptoms (Yin et al. 2019). Our findings indicated that the tested potato and tobacco miRNAs and their targets responded to PVY challenge (Yin et al. 2017, 2019); and the alteration patterns of these PVY-responsive miRNAs were host- and strain-dependent and may relate to symptom severity in PVY-host interaction.

In this study, we have two objectives. First, it is to demonstrate the chromosome location of the putative gene *Ny-DG* in the diploid potato clone DG 81-68, which exhibits a symptomless resistance to PVY^{NTN} at 20 °C and overcomes the resistance and development of necrotic lesions in both the inoculated leaves and the non-inoculated upper leaves at 28 °C. Second, it is to test how the selected PVY-responsive potato miRNAs (i.e. stu-miR162, stu-miR168a, stu-miR172e and two members of stu-miR482) and their putative target mRNAs (i.e. *DCL1*, *AGO1-2*, *TOE3*, *Gpa2* and *Cc-nbs-lrr*) would express in the DG 81-68 plants showing temperature-dependent response to PVY. We report for the first time the accumulation of miRNAs and target mRNAs in both the inoculated leaves and the non-inoculated upper leaves of a diploid potato inoculated with PVY^{NTN} in temperature-dependent reactions. The expression of five miRNAs and the corresponding putative target mRNAs were analysed based on their predicted function according to Xie et al. (2011) and Zhang et al. (2013). Amongst them, stu-miR162, stu-miR168a and their targets are supposed to be involved in miRNA biogenesis, plant development and stress responses. Stu-miR172e and its target *TOE3* are supposed to be involved in the development, defence response to virus, fungus and bacterium and response to heat. Stu-miR482 and its targets have been shown to be involved in defence response, incompatible interaction and plant-type HR. In addition, these five pairs were chosen because they are PVY-responsive and their expression was altered in potato cv. Etola challenged by PVY as shown in our previous work (Yin et al. 2017).

Materials and Methods

Plant Material

Diploid potato (*Solanum tuberosum* L., $2n = 2x = 24$) parental clones DG 81-68 and DW 83-3121 were crossed. The progeny consisted of 114 F1 individuals. The female parent DG 81-68 was resistant to PVY and had the pedigree described in Zimnoch-

Guzowska et al. (2000). The PVY-susceptible male parent DW 83-3121 was derived from inter-crossing *S. tuberosum* with wild potato species. Potato breeding clone PW 363 and potato cv. Rywal were used as controls carrying the gene *Ry-f*$_{sto}$ for ER resistance (Flis et al. 2005) and *Ny-1* for HR resistance (Szajko et al. 2008) to PVY inoculation, respectively.

PVY Inoculation at 20 and 28 °C and Collection of Leaf Samples

The PVY^{NTN} isolate 12-94 (GenBank: AJ889866.1) obtained from the IHAR-PIB Młochów virus collection was used. A sap extract (using autoclaved distilled water as the extraction buffer) from the plants of tobacco cv. Samsun infected with PVY^{NTN} was used as the inoculum. Plants of the diploid potato clone DG 81-68 in the 6–7 leaf stage were used for mechanical inoculation. For each plant, infectious leaf sap was applied to the lower three leaves and lightly sprinkled with carborundum powder; these lower three leaves were referred to as the inoculated leaves in this study. The leaves above these three inoculated leaves were referred to as the non-inoculated upper leaves. For each plant, small pieces of the three inoculated leaves, as well as small pieces of the three non-inoculated upper leaves, were sampled at 0.5 h post-inoculation (hpi) and at 3- and 6-day post-inoculation (dpi). Samples were stored at − 80 °C for RNA extraction.

The mechanically inoculated potato plants were divided into two groups; one group was incubated at 20 °C, the other at 28 °C, in the growth chambers under controlled environmental conditions (16 h light at 100 mol/s/m^2, 8 h dark). Mock-inoculated (inoculation with water) plants were used as controls. Experiments were repeated three times using three plants in each test.

Mapping of the Locus *Ny-DG*

The mapping population DG 81-68 × DW 83-3121 was used to find the chromosomal localization of the gene *Ny-DG*. In the mapping experiments, the presence of PVY was assayed in the parental clones and the F1 individuals which were grown at 20 °C by an ELISA using PVY monoclonal cocktail Bioreba AG kit (Reinach, Switzerland) as described previously (Szajko et al. 2014). The inoculated leaves and the non-inoculated upper leaves were tested by ELISA at 1 and 4 weeks post-inoculation, respectively. At 28 °C, the F1 progeny plants were evaluated for the appearance of necrotic symptoms in both the inoculated leaves and the non-inoculated upper leaves following PVY inoculation at 9 dpi. In addition, 2–4 tubers were collected from each plant inoculated at 20 °C and subsequently planted to examine PVY resistance of tuber progeny plants. The linkage group IX of parent DG 81-68 was constructed by scoring markers SC895 (Szajko et al. 2008) and TG591 (Szajko et al. 2014) linked to the gene *Ny-1*. Genetic distance between the marker loci and the locus *Ny-DG* was calculated as fraction of recombinants.

RNA Extraction, Reverse Transcription and Real-Time qPCR

RNA extraction and real-time RT-qPCR were conducted essentially according to Yin et al. (2017). Briefly, leaf samples were collected at 0.5 hpi and at 3 and 6 dpi from

PVY- and mock-inoculated (i.e. mechanical inoculation with water) plants of DG 81-68. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) in combination with DNase I digestion (DNA-free Kit, Ambion). RNA concentration and quality were measured with a spectrophotometer (Eppendorf BioSpectrometer).

Reverse transcription was conducted using 1 μg of total RNA with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The reverse transcription was conducted with the same condition and reagents for miRNA and mRNA. Real-time qPCR was conducted using SYBR Select Master Mix (Applied Biosystems) with a LightCycler480 real-time PCR instrument (Roche Diagnostics). The same reference gene, namely potato β-tubulin encoding gene (*TUB*, accession number Z33402), was used for the relative quantification of miRNA as well as for mRNA according to Yin et al. (2017).

In this study, the term quantification cycle (Cq) is used to indicate the fractional qPCR cycle used for quantification as used in Yin et al. (2017). The Cq is defined as the number of cycles at which the fluorescence signal exceeds a specific threshold level of detection. The raw Cq values for each gene in each sample were normalised to that of the reference gene (potato *TUB* gene) using the Advanced Relative Quantification method of the LIGHTCYCLER 480 software package. The software automatically calculates the relative expression levels (RELs) for each assay and displays it as $2^{-\Delta Cq}$. All ΔCq values were calculated as $Cq_{(analysed\ gene)} - Cq_{(reference\ gene)}$. The REL ($2^{-\Delta Cq}$) for each gene represented the mean of three biological replicates, where each replicate represented a mean of three technical replicates. Differences in the RELs ($2^{-\Delta Cq}$) of each gene between the PVY-inoculated and the mock-inoculated (inoculation with water) control samples were analysed by one-way ANOVA and multiple range test using Statgraphics Plus software. Expression change is shown as the ratio of $2^{-\Delta Cq}{}_{(PVY\text{-}inoculated\ sample)}/2^{-\Delta Cq}{}_{(mock\text{-}inoculated\ sample)}$ at each time point.

The expression levels of five potato miRNAs, namely stu-miR162, stu-miR168a, stu-miR172e, stu-miR482 (PotatoMir1005658171_x16170) and stu-miR482 (PotatoMir1005353123_x15854), were quantified by real-time stem-loop RT-qPCR, a method that allows two miRNAs with only a single nucleotide change to be differentiated using specific stem-loop primers (Chen et al. 2005). Their mRNA targets, namely *DCL1*, *AGO1-2*, *TOE3*, *Gpa2* and *Cc-nbs-lrr*, respectively, and the viral RNA PVY *HC-Pro* were tested by real-time RT-qPCR. Determination of the mRNA targets of the tested miRNAs was according to previous methods (Xie et al. 2011; Zhang et al. 2013). The predicted functions of the selected miRNAs/mRNA targets were defined in reference to Yin et al. (2017). The stem-loop primers specific for miRNAs, the primers specific for the mRNA targets and the primers for PVY *HC-Pro* RNA were chosen according to Yin et al. (2017).

Results

Reaction of DG 81-68 to Infection with PVY^{NTN} at 20 and 28 °C

The temperature-dependent development of symptoms and accumulation of the viral RNA in the leaves of the diploid potato clone DG 81-68 were observed. At 20 °C, no symptoms were observed in the inoculated leaves that were mechanically inoculated with PVY^{NTN} or in the non-inoculated upper leaves. At 28 °C, necrotic lesions were observed

in the inoculated leaves of DG 81-68 plants at 4–6 dpi (Fig. 1), and at 7 to 9 dpi, the necrotic symptoms were visualised in the non-inoculated upper leaves. When the plants of DG 81-68 that were inoculated and kept at 20 °C for 14 days were transferred to 28 °C, the appearance of necrotic lesions and the systemic spread of PVY occurred.

Using real-time RT-qPCR for PVY *HC-Pro* RNA according to Yin et al. (2017), low levels of the viral RNA were detected in the inoculated leaves at 0.5 hpi and 3 dpi at both 20 and 28 °C; PVY was not detected in the upper non-inoculated leaves in the corresponding experiments. At 6 dpi, high levels of *HC-Pro* RNA were detected in both the inoculated and the non-inoculated upper leaves at 28 °C, whereas the viral RNA was not detected in DG 81-68 at 20 °C (Fig. 1).

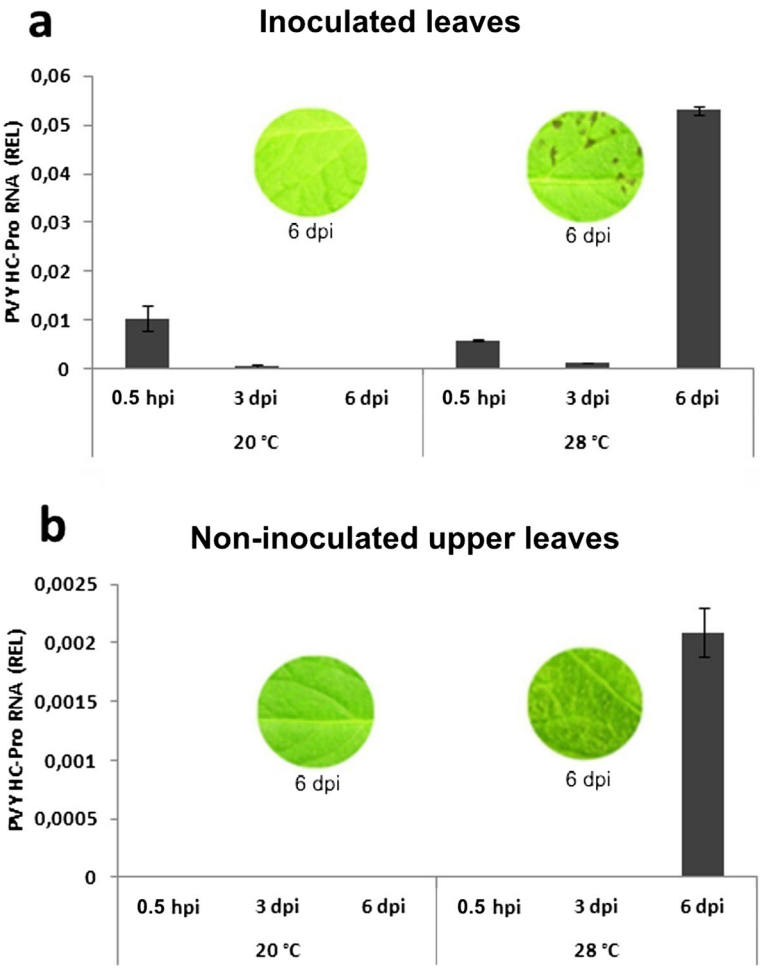


Fig. 1 Quantification of PVY *HC-Pro RNA* in both the inoculated leaves and the non-inoculated upper leaves of the diploid potato clone DG81-68 at 20 and 28 °C following PVY^{NTN} inoculation. hpi: hours post-inoculation; dpi: days post-inoculation; REL: relative expression level based on real-time RT-qPCR displayed as $2^{-\Delta Cq}$; the ΔCq value was calculated as $Cq_{\text{(analysed gene)}} - Cq_{\text{(reference gene)}}$. The reference gene used was potato β-tubulin encoding gene (*TUB*, accession number Z33402). Error bars represent standard deviation. Effect of temperature on the development of symptoms in the leaves of DG 81-68 plants at 6 dpi is shown

Location of the Gene *Ny-DG* on Potato Chromosome IX

An ELISA assay was used for the detection of PVY in mapping experiments at 20 °C. The PVY resistant parent DG 81-68 had very low ($A_{405} < 0.05$) and the susceptible parent DW 83-3121 had very high ($A_{405} > 1.4$) absorbance values according to ELISA tests in both the inoculated leaves and in the non-inoculated upper leaves of plants grown at 20 °C after 1 and 4 weeks post-inoculation, respectively.

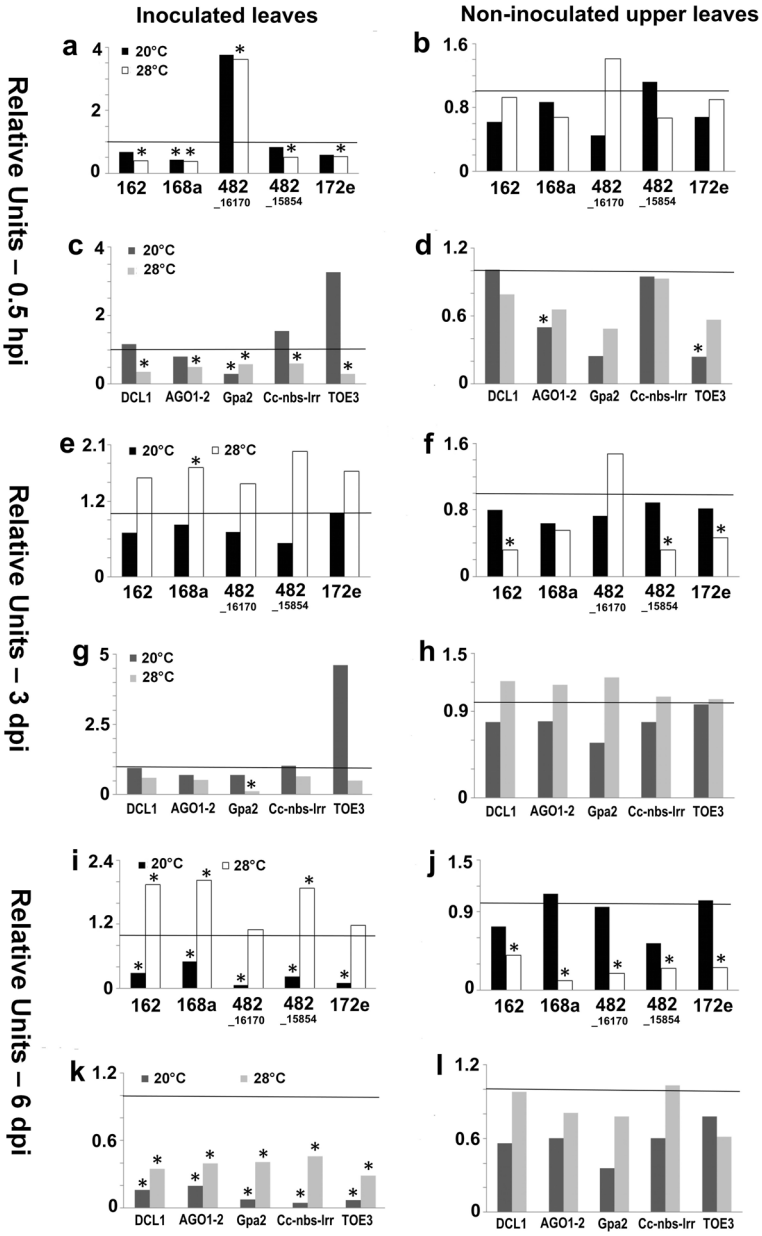
In the corresponding tests for the mapping population of DG 81-68 × DW 83-3121, out of the 114 F1 individuals, 51 plants were resistant (group A, having $A_{405} < 0.05$) and 63 were susceptible (group B, with A_{405} ranging from 0.15 to 1.6) to PVY^{NTN}. In the secondary infection assay for the plants from group A, PVY was not detected in all tuber progeny plants. In addition, at 28 °C, necrotic symptoms were observed in group A at 9 dpi, which confirmed the similar reaction type as the resistant parental clone DG 81-68.

In the mapping population DG 81-68 × DW 83-3121, the 1:1 segregation ratio of resistance versus susceptibility ($\chi 2 = 1.26$, $P = 0.26$) indicates the presence of a single, dominant gene present in the heterozygous state in the resistant parent DG 81-68. The two markers SC895 and TG591 were positioned 2.6 cM from the locus *Ny-DG*. This result confirms the location of *Ny-DG* on the long arm of potato chromosome IX.

Expression Changes of the miRNAs and Their Targeting mRNAs in the Leaves of DG81-68

In total, five miRNA/mRNA target pairs, namely stu-miR162/*DCL1*, stu-miR168a/*AGO1-2*, stu-miR172e/*TOE3*, stu-miR482 (PotatoMir1005658171_x16170)/*Gpa2* and stu-miR482 (PotatoMir1005353123_x15854)/*Cc-nbs-lrr*, were tested by real-time (stem-loop) RT-qPCR. The raw Cq values for each gene were normalised to that of the reference gene (potato *TUB* gene). The use of *TUB* as the reference gene for normalisation is according to Yin et al. (2017), and its expression stability in the tested tissue of the mock- and PVY-infected plants was assessed amongst 12 putative reference genes (Z. Yin, unpublished data). Differences in the REL of each gene between the PVY-inoculated and the mock-inoculated (inoculation with water) control samples were analysed by one-way ANOVA and multiple range test using Statgraphics Plus software. Expression change is shown as the ratio of REL of the PVY-inoculated sample and that of the mock-inoculated one for each gene at each time point at each temperature, and the results are presented in Fig. 2.

In a small number of cases, PVY infection led to significant changes in the expression levels of the tested miRNAs and their mRNA targets at 0.5 hpi (Fig. 2a–d) and 3 dpi (Fig. 2e–h). In the inoculated leaves at 0.5 hpi, downregulation of stu-miR168a (0.4-fold) and *Gpa2* (0.3-fold) was observed at 20 °C, and downregulation of all of the tested miRNAs and mRNAs was observed at 28 °C with the exception of stu-miR482 (PotatoMir1005658171_x16170), which was upregulated 3.76-fold (Fig. 2a, c). In the non-inoculated upper leaves at 0.5 hpi, downregulation of *AGO1-2* (0.5-fold) and *TOE3* (0.24-fold) was detected at 20 °C (Fig. 2d). At 3 dpi, upregulation of stu-miR168a (1.74-fold) and downregulation of *Gpa2* (0.13-fold) in the inoculated leaves were observed at 28 °C (Fig. 2e, g). In the non-inoculated upper leaves, downregulation of miR162 (0.3-fold), stu-miR482 (PotatoMir1005353123_x15854) (0.3-fold) and miR172e (0.5-fold) was detected at 3 dpi at 28 °C (Fig. 2f).



At 6 dpi, different expression patterns in the tested miRNAs and their targets were detected not only at different temperatures in plants showing symptomless or necrotic reaction but also at the same temperatures in the inoculated leaves compared with the non-inoculated upper leaves. In the inoculated leaves at 6 dpi at 20 °C, parallel downregulation occurred in all five tested miRNAs and their targets (Fig. 2i, k). At 6 dpi at 28 °C, the inoculated leaves, which possessed high amounts of the viral *HC-Pro* RNA (Fig. 1a), presented antagonistic expression of stu-

Fig. 2 miRNA and mRNA target accumulation levels in both the inoculated leaves and the non-inoculated upper leaves of the PVY^{NTN}-inoculated diploid potato clone DG 81-68 at 20 °C (symptomless response) and at 28 °C (necrotic lesions) by real-time (stem-loop) RT-qPCR. The tested five miRNA/mRNA target pairs are stu-miR162/*DCL1*, stu-miR168a/*AGO1-2*, stu-miR172e/*TOE3*, stu-miR482 (PotatoMir1005658171_x16170)/*Gpa2* and stu-miR482 (PotatoMir1005353123_x15854)/*Cc-nbs-lrr*. Expression change (relative units) for each gene is shown as the ratio of $2^{-\Delta Cq}{}_{(\text{PVY-inoculated sample})}/2^{-\Delta Cq}{}_{(\text{mock-inoculated sample})}$ at each time point. The ratio value 1.0 is indicated by a horizontal line. The raw Cq values for each gene in each sample were normalised to that of the reference gene, potato β-tubulin encoding gene (*TUB*, accession number Z33402). The relative expression level (REL) for each assay is displayed as $2^{-\Delta Cq}$. All ΔCq values were calculated as $Cq_{(\text{analysed gene})} - Cq_{(\text{reference gene})}$. The REL ($2^{-\Delta Cq}$) for each gene represented the mean of three biological replicates, where each replicate represented a mean of three technical replicates. Differences in the REL ($2^{-\Delta Cq}$) of each gene between the PVY-inoculated and the mock-inoculated (inoculation with water) control samples were analysed by one-way ANOVA and multiple range test using Statgraphics Plus software. Differences were assumed to be statistically significant at p value ≤ 0.05. dpi: days post-inoculation; hpi: hours post-inoculation; post-inoculation; 168a: stu-miR168a; 162: stu-miR162 (PotatoMir1005244514_x19366); 482_16170: PotatoMir1005658171_x16170; 482_15854: PotatoMir1005353123_x15854; 172e: stu-miR172e; AGO1-2 (PGSC0003DMC400054073): isoform 2 of Argonaute 1; DCL1 (GSC0003DMT400029301): endoribonuclease Dicer homologue 1; TOE3 (CK265044): apetala2-like ethylene-responsive transcription factor TOE3-like; Gpa2 (PGSC0003DMT400050510): disease resistance protein Gpa2; Cc-nbs-lrr (PGSC0003DMT400019599): cc-nbs-lrr resistance protein, JHL06P13.14 protein. *Statistically significant differences compared with the control (p value ≤ 0.05)

miR162, stu-miR168a and stu-miR482 (PotatoMir1005353123_x15854) with their targets; thus, the increased expression of these miRNAs promoted the downregulation of their targets. Stu-miR162, stu-miR168a and stu-miR482 (PotatoMir1005353123_x15854) were highly upregulated and presented levels 1.9-, 2.0- and 1.9-fold compared with the levels observed in the mock-inoculated controls, respectively (Fig. 2i). In addition, the corresponding target mRNAs, *DCL1*, *AGO1-2* and *Cc-nbs-lrr*, were decreased by 0.35-, 0.40- and 0.46-fold compared with the controls, respectively (Fig. 2k). For stu-miR482 (PotatoMir1005658171_x16170) and stu-miR172e, although their levels were downregulated at 20 °C, their expression remained unchanged at 28 °C at 6 dpi, and downregulation of their target mRNAs *Gpa2* (0.4-fold) and *TOE3* (0.3-fold) occurred at 28 °C at 6 dpi in the inoculated leaves (Fig. 2i, k). At 6 dpi at 20 °C, the non-inoculated upper leaves, which contained no detectable viral *HC-Pro* RNA, did not show changes in the expression levels of the tested miRNAs or their targets (Fig. 2j, l). In the corresponding experiments at 28 °C, the downregulation of all five miRNAs was correlated with high levels of the viral *HC-Pro* RNA in the non-inoculated upper leaves at 6 dpi, whereas the expression levels of their targets remained unchanged (Fig. 2j, l).

Discussion

Early findings have demonstrated variants of temperature-dependent HRs in potato. The hypersensitivity gene *Ny-1* confers resistance to PVY in potato cv. Rywal. The virus was localised via HR when the plants were grown at 20 °C, whereas the plants were systemically infected but no symptoms were observed at 28 °C (Szajko et al. 2008). Similar results were obtained in the potato cvs. Albatros and Sekwana, which possess the genes *Ny-1A* and *Ny-1S*, respectively (Szajko et al. 2014). However, the potato cv. Sárpo Mira showed HR to PVY at

both 20 and 28 °C (Tomczyńska et al. 2014). In this study, the potato diploid clone DG 81-68 exhibited necrotic lesions in both the inoculated leaves and the non-inoculated upper leaves to PVY infection at 28 °C. At 20 °C, the virus was localised in the inoculated leaves without symptoms, such as in PW 363 plants possessing *Ry-f*$_{sto}$ which is the gene for the ER response. Compared with the observations in PW 363 (Flis et al. 2005), the systemic spread of PVY in DG 81-68 occurred when the plants that were inoculated and kept at 20 °C for 14 days were transferred to 28 °C. The temperature-dependent reaction observed in DG 81-68 was different from that observed in potato cv. Rywal possessing the temperature-dependent HR resistance gene *Ny-1* to PVY. In cv. Rywal, necrotic lesions were observed in the inoculated leaves and no symptoms in the non-inoculated upper leaves, and the virus was localised when plants were grown at 20 °C, whereas at 28 °C, plants were systemically infected but no symptoms were observed in both the inoculated leaves and the non-inoculated upper leaves (Szajko et al. 2008). In the plants of DG 81-68 following PVY^{NTN} inoculation at 20 °C, no symptoms were observed and no viral RNAs were detected in the non-inoculated upper leaves, which indicates DG 81-68 displays symptomless resistance to PVY^{NTN}. However, at 28 °C, the necrosis observed in both the inoculated leaves and the non-inoculated upper leaves and systemic spreading of viral RNA indicate overcoming the resistance. *Ny-DG* is the first reported *R* gene for PVY resistance of its type in potato. The genes *Ny-1*, *Ny-1A* and *Ny-1S* were mapped on the long arm of the potato chromosome IX (Szajko et al. 2008, 2014), and their genetic positions correspond to the location of *Ny-Smira* in the potato cv. Sárpo Mira (Tomczyńska et al. 2014). The locus *Ny-DG* was also mapped to this chromosome region. It is likely that all of these resistance genes belong to the same *R* gene cluster and represent different alleles at the same locus.

We analysed the expression levels of five host miRNAs and their targets in DG 81-68 plants showing symptomless resistance at 20 °C and necrotic lesions at 28 °C after PVY^{NTN} inoculation. The results obtained indicated that the expression patterns of the tested miRNAs and target mRNAs were altered differently not only at different time points post-inoculation but also in the inoculated and upper leaves, and their accumulation levels were related to the type of reaction, which in turn was dependent on temperature.

Alteration in the expression levels of the miRNAs and their targets seen in this study was also observed in other plant species upon virus infection. Stu-miR482 has been suggested to be involved in the regulation of *NB-LRR*-type disease-resistance *R* genes in plants (Shivaprasad et al. 2012; de Vries et al. 2015). The downregulation of miR482 and upregulation of *NB-LRR* transcripts at the infection sites were observed in tomato plants infected with turnip crinkle virus (TCV), cucumber mosaic virus (CMV) and tobacco rattle virus (TRV) (Shivaprasad et al. 2012) and in cowpea (*Vigna unguiculata* (L.) Walp.) plants infected with groundnut bud necrosis virus (GBNV) (Permar et al. 2014). Previous studies have indicated that miR172 might be linked to leaf curl disease in tomato caused by tomato leaf curl New Delhi virus infection (Naqvi et al. 2010). In *N. tabacum* plants, TMV infection causes the downregulation of miR172 at an early stage and upregulation at a later stage (Bazzini et al. 2007, 2011). Plant DCL1 and AGO1 represent two key enzymes in the miRNA biogenesis pathway, and they are regulated by miR162

and miR168, respectively (Xie et al. 2003; Vaucheret et al. 2004; Voinnet 2009). Induction of miR168 and its target *AGO1* mRNA is commonly observed in plant-virus interactions (Yin et al. 2014). In soybean genotype PI96983 carrying the strain-specific resistance gene *Rsv1*, highly elevated levels of miR168 and *AGO1* mRNA were detected only in G7-infected *Rsv1* plants showing a lethal systemic hypersensitive response (LSHR). However, in the *Rsv1* plants resistant to strain G2, no significant difference was found in expression of miR168 compared to the mock-inoculated control (Chen et al. 2015).

In potato, in our previous study, the expression of the same group of miRNAs and targets has been analysed in cv. Etola showing strain-specific HR resistance to PVY. In the non-inoculated upper leaves of the plants of cv. Etola, parallel increases in the expression levels of stu-miR168, stu-miR162, stu-miR172e and two members of stu-miR482, together with their targets *AGO1-2*, *DCL1*, *TOE3*, *Gpa2* and *Cc-nbs-lrr*, respectively, were observed in $PVY^{N\text{-}Wi}$-infected plants showing necrotic reaction and severe symptoms. However, changes were not observed in the levels of the same set of miRNAs and their targets in PVY^{NTN}-inoculated plants showing HR resistance or in PVY^{Z}-NTN-infected Etola showing necrotic reaction and mild symptoms (Yin et al. 2017). In another example, potato cv. Rywal carrying *Ny-1* gene exhibited temperature-dependent HR resistance to PVY at 20 °C (Szajko et al. 2008). The transcriptional reprogramming was pronounced in the inoculated leaves of the plants of cv. Rywal following PVY inoculation at 1 and 3 dpi at 20 °C, including the downregulation of genes related to RNA silencing, for example, *AGO1* transcript (Baebler et al. 2014). Regulation of immune receptor transcripts by miR6022 as well as upregulation of miR164, miR167, miR169, miR171, miR319, miR390 and miR393 in potato cv. Désirée showing tolerant response to PVY^{NTN} revealed similarities to responses observed in mutualistic symbiotic interactions (Križnik et al. 2017).

In this study, in the non-inoculated upper leaves of potato DG 81-68 plants following PVY^{NTN} inoculation at 6 dpi, the downregulation of the tested miRNAs was detected at 28 °C in plants showing necrosis in the inoculated leaves but not at 20 °C in plants showing symptomless resistance. Their corresponding targets remained unchanged at both temperatures. Moreover, in the inoculated leaves of potato DG 81-68 plants following PVY^{NTN} inoculation at 6 dpi, parallel downregulation of the tested miRNAs and their targets were observed at 20 °C in plants showing symptomless resistance. However, the increased expression of the tested miRNAs was detected, and the levels of their target transcripts were decreased at 28 °C in plants showing necrotic lesions. The downregulation of miRNAs in the non-inoculated upper leaves observed in this study shares similarities with the pattern of alterations in a group of biotic and abiotic stress-responsive miRNAs in the early stage of TMV infection in tobacco as demonstrated by Bazzini et al. (2011). The authors inferred that the basal defence and the signals of viral-associated molecular pattern (VAMPs) might play a role in the early stages of miRNA alteration (Bazzini et al. 2011). As for the upregulation of miRNAs in the inoculated leaves observed in this study (3 and 6 dpi), it is similar to that observed in Désirée-PVY^{NTN} tolerant response (Križnik et al. 2017). The authors demonstrated the downregulation of gibberellin signalling at 3 dpi in the inoculated leaves before viral multiplication could be detected and it might be linked to the reduced disease severity. Moreover, the

discordance between miRNA and mRNA expression patterns observed in the non-inoculated upper leaves of DG 81-68 may indicate that additional miRNAs may regulate the same target transcript. For example, *TOE3* is targeted by stu-miR172a and stu-miR1533a in addition to stu-miR172e, and *AGO1-2* is targeted by stu-miR1522c besides stu-miR168a (Zhang et al. 2013). The alteration of the tested potato miRNAs and their targets was strain-dependent and related to symptom severity in the PVY-Etola interactions. In the PVY-DG 81-68 interactions, changes in the same tested set of potato miRNAs and their targets expression levels were temperature-dependent and related to the reaction type and differed between the inoculated leaves and the non-inoculated upper leaves.

In summary, a few miRNAs and their targets were tested in DG 81-68-PVY^{NTN} interaction. However, the alteration patterns of their expression showed a clear tendency of depending on the site of infection and temperature. This data will lay the foundation for our further study on the role of miRNA in potato-PVY interaction at large scale and at genome level. It might also be useful information for researchers interested in similar subjects in other plant species.

Author Contribution K.S. carried out phenotyping and performed the genetic and molecular studies. Z.Y. participated in the design of the molecular studies, performed the statistical analysis and co-wrote the paper. W.M. conceived and coordinated the project, and co-wrote the paper. All authors reviewed the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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