Expression of the Potato Late Blight Resistance Gene *Rpi-phu1* and *Phytophthora infestans* Effectors in the Compatible and Incompatible Interactions in Potato

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ABSTRACT

This study describes late blight resistance of potato breeding lines resulting from crosses between cultivar ‘Sárpo Mira’ and *Rpi-phu1* gene donors. The progeny is investigated for the presence of *Rpi-Smira1* and *Rpi-phu1* resistance (*R*) genes. Interestingly, in detached-leaflet tests, plants with both *R* genes withstood the infection of the *Phytophthora infestans* isolate virulent to each gene separately, due to either interaction of these genes or the presence of additional resistance loci. The interaction was studied further in three chosen breeding lines on the transcriptional level. The *Rpi-phu1* expression, measured over 5 days, revealed different patterns depending on the outcome of the interaction with *P. infestans*: it increased in infected plants whereas it remained low and stable when infection was

unsuccessful. The expression patterns of *P. infestans* effectors *Avr-vnt1*, *AvrSmira1*, and *Avr8*, recognized by the *Rpi-phu1*, *Rpi-Smira1*, and *Rpi-Smira2* genes, respectively, were evaluated in the same experimental setup. This is the first report that the *Avr-vnt1* effector expression is not switched off permanently in virulent isolates to avoid recognition by an *R* protein but can reappear in a postbiotrophic phase and is present constantly when infecting plants without the corresponding *R* gene. Both a plant and a pathogen can react to the other interacting side by changing the transcript accumulation of *R* genes or effectors.

Additional keywords: *Solanum tuberosum*.

Phytophthora infestans is the oomycete pathogen that causes late blight of potato (*Solanum tuberosum* L.). This economically important disease can decrease global potato production by approximately 16%, representing financial loss of U.S.\$7.2 billion per annum (Haverkort et al. 2009). Fungicides may be used to control the disease; however, their application is expensive and not always effective because fungicide-resistant strains might emerge. The discovery of the main late blight resistance (*R*) genes from *S. demissum* (*R1* to *R11*) (Black et al. 1953) led to their application in potato breeding programs. More than 40 *R* genes against late blight were identified and their chromosomal positions were determined within the last two decades (Śliwka and Zimnoch-Guzowska 2013).

The nucleotide-binding site leucine-rich repeat (NBS-LRR) domain has been found in most *R* genes, including potato genes for late blight resistance (Eitas and Dangl 2010). *R* proteins are responsible for the recognition of pathogens proteins called effectors, which lead to the hypersensitive reaction (HR) and prevention of the infection. The *Rpi-phu1* gene provides very high resistance against late blight, represents the NBS-LRR gene family, and was mapped to the potato chromosome IX (Śliwka et al. 2006). *Rpi-vnt1.1* from *S. venturii* was mapped to the same region and it was revealed that nucleotide sequences of both *R* genes are identical (Foster et al. 2009). As reported by Brylińska et al. (2016), only 3% of 319 *P. infestans* isolates collected between 2010 and 2012 in selected regions of Poland were able to overcome the resistance conferred by the *Rpi-phu1* gene. The *Rpi-phu1* gene was introgressed from an

interspecific hybrid *S. phureja* × *S. stenotomum* to a diploid *S. tuberosum* (Śliwka et al. 2006). Some differences in expression of this gene were observed between plants of different genetic background (Śliwka et al. 2013). Because no cultivar with the *Rpi-phu1* gene has been yet released, there is little selection pressure on pathogen population against that gene.

Only a few potato cultivars are resistant to *P. infestans*. ‘Sárpo Mira’, registered in the United Kingdom in 2002, still retains high late blight resistance (White and Shaw 2009). Studies concerning a broad-spectrum resistance of Sárpo Mira revealed the presence of at least four major *R* genes (*R3a*, *R3b*, *R4*, and *Rpi-Smira1*) and the *Rpi-Smira2* locus conferring quantitative resistance (Rietman et al. 2012). *Rpi-Smira1* is located on chromosome XI of the potato genome near the *R3* gene cluster, where markers with strong linkage to the resistance locus were found (Tomczyńska et al. 2014). *Rpi-Smira2* has been colocalized with the *R8* locus and both loci conferred similar resistance levels. Additionally, the *P. infestans* effector *Avr8* was reported to trigger an HR response of the *Rpi-Smira2* gene in Sárpo Mira. Thus, it was suggested that *R8* and *Rpi-Smira2* are identical or functional homologs (Jo 2013).

Effectors secreted by plant pathogens alter the function and structure of a host cell and, thereby, facilitate infection (Kamoun 2006). The genes coding effectors are located in gene-sparse, transposon- and repeat-rich regions of the *P. infestans* genome (Haas et al. 2009), therefore undergoing frequent mutations (van Damme et al. 2011). Modified forms of the effectors may evade recognition by *R* genes due to single mutations, resulting in proteins with changed amino acids sequences. Such an evasion strategy is documented for the *Avr3a* effector: the *Avr3a^{K1}* protein is normally recognized by *R3a*; however, the allelic form with two amino acids changes (*Avr3a^{EM}*) remains invisible for the corresponding *R* gene (Armstrong et al. 2005). An allelic variant with a premature stop codon of *Avr4* effector codes a truncated version of *Avr4* protein which also remains unrecognized by potato plants with the *R4* gene (van Poppel et al. 2008). Another evasion strategy is associated with an expression pattern of effectors. The cytoplasmic effector *Avr-vnt1* is expressed in a biotrophic phase of infection and recognized by plants with the *Rpi-vnt1.1/Rpi-phu1* *R*

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gene. In the virulent isolates, no transcript of *Avr-vnt1* effector was found, even though an intact gene was present (Pel 2010). A similar strategy has been reported for *Avr2*. This effector, once expressed, is recognized by the *R2* gene but no expression of the *Avr2* was reported in the virulent isolates. The virulent *P. infestans* isolates possess an additional *Avr2* copy, called *Avr2-like*, which is different in 13 amino acids and is expressed in virulent strains. The *Avr2-like* effector remains undetected in *R2* plants (Gilroy et al. 2011).

Few studies of *R* gene expression during an interaction with *P. infestans* isolates have been published. The expression of the *RB* gene has been followed in unchallenged plants (Iorizzo et al. 2011) and in compatible interactions with transgenic potato lines carrying the gene (Bradeen et al. 2009; Kramer et al. 2009; Millett et al. 2009). Huang et al. (2005) has studied the *R3a* gene expression measured in transgenic plants, and the *Rpi-phu1* transcript level was investigated by Śliwka et al. (2013) in nontransgenic plants, both in incompatible reactions.

In this study, we compared the differences in the *Rpi-phu1* *R* gene expression in potato hosts interacting with compatible and incompatible *P. infestans* isolates. To our knowledge, this is the first report investigating changes in transcript levels of the same *R* gene in interactions leading to either resistance or susceptibility. To select plant material, resistance against four *P. infestans* isolates was tested in the progeny resulting from crossing of Sárpo Mira (with *Rpi-Smira1*, *Rpi-Smira2*, *R3a*, *R3b*, and *R4* *R* genes) with two clones carrying gene *Rpi-phu1*. The results of resistance tests and molecular markers linked to the *R* genes were used to define the presence of the *Rpi-phu1* and *Rpi-Smira1* genes in the progeny. *Rpi-Smira2* might have also contributed to their resistance, whereas the applied *P. infestans* isolates avoided recognition by *R3a*, *R3b*, and *R4*. Three chosen potato genotypes, varied in their genetic background and *R* gene presence, were challenged with the same four *P. infestans* isolates. In order to test whether changes in effector transcript levels affected the interaction outcome, the expression patterns of three

P. infestans effectors—*Avr-vnt1*, *AvrSmira1*, and *Avr8*, recognized by the *Rpi-phu1*, *Rpi-Smira1*, and *Rpi-Smira2* genes, respectively—were also evaluated in the same experimental setup.

MATERIALS AND METHODS

Plant material and marker-assisted selection. Potato breeding lines ($n = 83$) resulting from crossing Sárpo Mira with the two donor clones Z-03.3817 and Z-03.3827, both carrying gene *Rpi-phu1*, were obtained by the Potato Breeding Zamarte Ltd.-IHAR Group (Tomczyńska et al. 2014). Progeny lines were tested with two molecular markers for the presence of *R* genes originating from both parents: marker 45/XI for the presence of *Rpi-Smira1* and *phu6* for the presence of *Rpi-phu1* (Table 1). The *phu6* primers were used in following polymerase chain reaction (PCR) conditions: 94°C for 3 min; 39 cycles of: 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; followed by 72°C for 7 min. The program for 45/XI markers consisted of 94°C for 3 min; 39 cycles of: 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min; followed by 72°C for 7 min. Three genotypes were chosen for the gene expression assay: 04-IX-21 (Śliwka et al. 2013) and Z11 with *Rpi-phu1* and genotype Z5 with *Rpi-phu1* and *Rpi-Smira1* *R* genes. The latter two resulted from a Sárpo Mira × Z-03.3817/Z-03.3827 cross. Susceptible ‘Craig’s Royal’ was used as a control.

***P. infestans* isolates.** Four *P. infestans* isolates from the collection of the Plant Breeding and Acclimatization Institute–National Research Institute in Młochów were chosen for detached-leaflet assays (DLA). The isolates were chosen according to their capability to overcome the resistance conferred by *Rpi-phu1* and *Rpi-Smira1* genes: isolate MP324 was avirulent, whereas MP1580 was virulent to both of the investigated *R* genes. MP324x and MP1498 were able to defeat the resistance conferred by single genes *Rpi-phu1* and *Rpi-Smira1*, respectively. Detailed characteristics of the isolates used are displayed in Table 2.

TABLE 1. List of primers used in this study, with references

Name	Primers 5'→3'	Product size (bp)	Accession numbers ^a	References
45/XI	AGAGAGGTTGTTCCGATAGACC TCGTTGTAGTTGTCTTCCACAC	900–1,500	PGSC chr11:39956309..39958308	Tomczyńska et al. 2014
phu6	AGAGACCCTGGATATATTTTCATAGCTCT CGCTCTAGGCACAGGGCTCAATGCTGAT	298	GB FJ423044	Śliwka et al. 2013
57-α-tubulin	AATTTGTCGACTGGTGTCTCT GTCAATGCGAGAGAAGACCT	152	GB NM_001288543	Kramer et al. 2009
Avr_n2	AAGCGAGCCGACTTTACAGC CCAGTCATCTGCGTACTTCG	156	GB XM_002897316	This study
AvrSmiraC	ACGATTGCTCACGAAAACCC GAGGAATTTGACATCGTCGTCC	175	GB XM_002904490	This study
Avr8_A	CACCAGCACCGCCACAAG GGTCTCTAACCAACCCTCACTT	152	GB XM_002904498	This study
Avr3a	GCGAATCTAAATGAGGAGATGTTTAAT (AR) CCTGTTGTGCTGCCACCAA (aR) CCTGTTGTGCTGCCACCAC (R) CCTGTTGTGCTGCCACCA	132	GB HQ639930.1	Clément et al. 2013
Ef2α	TGACGCTATCGCAAGGAATC GACTGACGCAACTGTCTCGG	216	GB XM_002901697	Torto et al. 2002 and this study
AvrVnt1	TCACACGCCACGCCGAGTA	737	GB PITG_16294	This study
whG_B	TCGCCAGCTTCGACAGAGC	...	...	...
AvrSmira1	CGATGTCTCCCAATTCTCATTTCC	853	GB PITG_07550	This study
whG_B-C	GTCGCACCGAACTCAACAATACTT	...	...	...

^a PGSC = Solanaceae Genomics Resource Genome Browser Potato (*Solanum phureja* clone DM1-3 516R44) PGSC v2.1.11 Pseudomolecules and GB = GenBank.

TABLE 2. Characteristics of *Phytophthora infestans* isolates used in the study

Isolate	Year, origin	Mating type	Race	<i>Rpi-phu1</i>	<i>Rpi-Smira1</i>
MP324	1997, Koszalin	A1	1.2.3.4.5.6.7.8.10.11	Avirulent	Avirulent
MP324x	2012, Młochów	A1	1.2.3.4.6.7.(10)0.11	Virulent	Avirulent
MP1498	2012, Boguchwała	A1	1.3.4.5.7.10.11	Avirulent	Virulent
MP1580	2012, Białuty	A1	1.3.4.5.7.10.11	Virulent	Virulent

DLA. Prior to laboratory tests, chosen *P. infestans* isolates were grown on leaves of susceptible ‘Tarpan’ or ‘Irys’ at least twice. Sporangia were rinsed with sterile water and their suspension was adjusted to a concentration of 5×10^4 /ml using a hemocytometer. Further incubation of inocula was carried out at 5°C for 3 h of stimulated zoospore release.

Resistance against each of four *P. infestans* isolates (Table 2) was tested on two different dates in two replications per date. Per replication, three lateral leaflets of each genotype were collected from young but fully developed leaves from 6-week-old greenhouse-grown plants. Leaflets were then put with the abaxial side up on wet blotting paper in a plastic box covered with glass to maintain humidity (Zarzycka 2001). Each leaflet was inoculated with two separate 30- μ l droplets using a pipette and incubated in a climatic chamber at 16°C in high relative humidity and darkness. The leaflets were turned over after 24 h and incubation continued in constant light, with other conditions preserved. Resistance was estimated after 7 days of incubation on a scale of 1 to 9, where 9 = no late blight symptoms visible (Zarzycka 2001).

Gene expression assay. Leaflets of genotypes 04-IX-21, Z11, Z5, and Craigs Royal, uniform in size and weighing approximately 100 mg, were used for the gene expression analysis in a DLA. Prior to inoculation, three leaflets of each genotype were collected for the basal expression measurements. The test was conducted on two dates and 15 leaflets of each genotype were inoculated with each of four *P. infestans* isolates used (Table 2). On each of five subsequent days, three inoculated leaflets were collected into 2-ml tubes, immediately frozen in liquid nitrogen, and stored at -80°C. Leaflet samples were ground in liquid nitrogen using polypropylene pestles. Total RNA was isolated using the Spectrum Plant Total RNA Kit and treated with the On-Column DNase I Digestion Set (Sigma-Aldrich, St. Louis) according to the manufacturer’s instructions. The quality and quantity of the isolated RNA were tested with a BioPhotometer spectrophotometer (Eppendorf AG, Hamburg, Germany) and its integrity was validated after the electrophoresis under UV light with ethidium bromide staining.

RNA (400 ng) was then used for reverse transcription with oligo (dT)₁₈ and random hexamer primers in 20- μ l reaction volumes using the Maxima First-Strand cDNA Synthesis Kit for reverse-transcription (RT)-PCR (Fermentas, Thermo Scientific, St. Leon-Rot, Germany). The RT-PCR program was run as follows: 10 min at 25°C of initial incubation, 15 min at 50°C of cDNA synthesis, and reaction termination by heating to 85°C for 5 min. cDNA concentrations were adjusted to 5 ng μ l⁻¹ and 2 μ l of cDNA was used as a template for quantitative (q)PCR. The qPCR mixtures were prepared in 20- μ l reaction volumes containing 10 μ l of the LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany), primers at 0.5 μ M each, and PCR-grade water. The primers used in the gene expression assays are listed in the Table 1 along with the accession numbers of the sequences their design was based on; effector primers are also shown in the alignments in Supplementary Figures S2 and S4. The qPCR was performed in the LightCycler 480 II instrument (Roche Diagnostics) and each sample was analyzed in three technical replications. To exclude potential qPCR contaminations, three no-template controls were added to each run. The *Rpi-phul* gene expression levels were measured using the following program: 5 min at 95°C and 40 cycles of 95°C for 10 s, 65°C for 20 s, and 72°C for 30 s. A similar program was used for the relative quantification of effectors, with the annealing time shortened to 10 s at 65°C and number of cycles extended to 50. After amplification, PCR product melting was performed at a temperature range of 65 to 97°C with the resolution of five acquisitions per degree centigrade, and the resulting melting curves were analyzed to exclude potential unspecific products. Potato α -tubulin displaying consistent quantification cycles (Cq) was used as the reference gene in the *Rpi-phul* expression assay (Kramer et al. 2009) and *P. infestans* elongation factor 2- α (ef2 α) was used for the effector assays (Torto et al. 2002). To test the efficiency of newly designed primers (Avr_n2, AvrSmiraC, Avr8_A, and Ef2 α), standard

PCR were run with corresponding primer sets using the same cDNA template. The products were then purified with a GenElute PCR Clean-Up Kit (Sigma-Aldrich) and serial dilutions were prepared, to be used further in qPCR. Primer efficiencies were calculated for each of the primer sets. The threshold value was adjusted manually to 0.7 for each qPCR run. This value set in an exponential phase guaranteed extraction of data normalized between runs. Cq greater than 40 were ignored because they are thought to be unreliable. The data of the relative expression were analyzed in Microsoft Excel software using the $\Delta\Delta C_q$ method to enable comparisons between data from different experimental combinations (Livak and Schmittgen 2001). Primers efficiencies for the *AvrSmira1* and *Avr8* effectors were less than 2; therefore, the calculation of relative expression was performed using the mean efficiency of target and reference gene primer sets that was equal to 1.9, whereas the $2^{-\Delta\Delta C_t}$ formula was used in the case of the *Avr-vnt1* effector. Statistical analyses were performed using Statistica 10 software (Statsoft Inc. 2011).

To investigate the potential influence of stress conditions on *R* gene expression, an additional experiment was performed. Six-week-old plants of the 04-IX-21 genotype grown in controlled conditions (16 h of daylight at 18°C and 8 h of darkness at 16°C, with relative humidity between 80 and 90%) were sprayed with a sporangial suspension of the isolate MP324 (concentration of 5×10^4 /ml). As a susceptible control, plants of Craigs Royal were used. Leaflets from five biological replicates of the 04-IX-21 genotype were collected before and on each of five subsequent days postinoculation (dpi), frozen in liquid nitrogen, and processed further, as described in the DLA procedure. The *Rpi-phul* transcript levels in undetached and detached leaflets of the MP324-inoculated genotype 04-IX-21 were then compared.

The survey of the *Avr3a* effector was included in the experiments as an internal control of the effector assay. The DNA of MP324x and MP1580 and cDNA of these *P. infestans* isolates used for inoculation of Craigs Royal were investigated for the presence of the *Avr3a* gene fragments using two primer sets: *Avr3a-F/-AR* for avirulent and *Avr3a-F/-aR* for virulent alleles of the *Avr3a* effector (Table 1). For this purpose, a standard PCR with the qPCR program for the relative quantification of effectors was employed. The reaction was performed in a volume of 20 μ l with 0.1 μ M dNTP, 1 $\times$ PCR buffer, 0.5 μ M each primer, 1 U of *Taq* polymerase, and PCR-grade water. Products were examined on 1.5% agarose gel stained with ethidium bromide. The same qPCR program and reagent concentrations used for the *Avr-vnt1*, *AvrSmira1*, and *Avr8* effector fragment amplification were also applied to the *Avr3a* effector. The *Avr3a-F/-R* primer pair, yielding a product of 132 bp, was used (Table 1).

Effector alleles. The genomic DNA of four *P. infestans* isolates used in this study served as a template for amplification of the *Avr-vnt1* and *AvrSmira1* genes. The PCR primers based on the PITG_16294 and PITG_07550 reference sequences were designed using the Net Primer software (www.premierbiosoft.com/netprimer/). To obtain the 462-bp coding sequence of the *Avr-vnt1* gene, *AvrVnt1_whG_B* forward was used with either *AvrVnt1_whG_B* reverse or *AvrVnt1_n1* reverse primers, yielding a 737- and 529-bp product, respectively. The 717-bp coding sequence of *AvrSmira1* effector was obtained with the *AvrSmira1_whG_B-C* primer pair (Table 1). Detailed information on the reaction conditions can be found in Supplementary Table S2. PCR products were validated with ethidium bromide staining under UV light, purified with the GenElute PCR Clean-Up Kit (Sigma-Aldrich), and sequenced by an external company (Genomed S.A., Warsaw, Poland). The ChromasLite 2 software (http://technelysium.com.au/wp/) was used to proofread and trim sequences, whereas single-nucleotide polymorphisms (SNP) were investigated with NovoSNP software (Weckx et al. 2005). To resolve ambiguous sequences, computational phasing was performed using PHASE 2.1 (Stephens and Donnelly 2003; Stephens et al. 2001). Effector sequences were processed further in the SeaView 4 software (Gouy et al. 2010).

RESULTS

Late blight resistance in a DLA. Based on the DLA for resistance to late blight and PCR markers for the *Rpi-phul* and *Rpi-Smira1* genes, of 83 potato lines, 24 were assigned to group 1 (with none of the investigated genes), 21 to group 2 (*Rpi-Smira1* only), 31 to group 3 (*Rpi-phul* only), and 7 to group 4 (both investigated genes present). Four *P. infestans* isolates used in the DLA differed in their virulence (Table 2). Regardless of the isolate used, the lowest mean resistance was observed in lines of group 1. Group 2 displayed high resistance against isolates MP324 and MP324x, whereas group 3 was resistant to MP324 and MP1498. The highest mean resistance was observed in lines of group 4 (Fig. 1), with mean rating between 7.9 (inoculation with MP324x) and 8.9 (MP324). None of the tested isolates overcame the resistance in the lines containing *Rpi-phul* and *Rpi-Smira1* (group 4), even though isolate MP1580 was able to defeat resistance conferred by each gene present separately. Inoculation with isolate MP324 distinguished three resistant groups (groups 2, 3, and 4); however, mean scores of groups 3 and 4 were significantly higher than the score of group 2 (Fig. 1A). The same levels of resistance to the isolate MP324x were revealed in groups 2 and 4

of the tested potato lines, whereas no significant differences were observed between susceptible groups 1 and 3 (Fig. 1B). Although lines of both group 1 and 2 were considered susceptible, the latter performed significantly better when challenged with isolate MP1498 (Fig. 1C). When *P. infestans* isolate MP1580 was used for inoculation, three susceptible groups were revealed, with groups 2 and 3 displaying mean scores significantly higher than those of group 1 (Fig. 1D).

The marker-assisted selection (MAS) results were compiled with the phenotypes observed in the DLA. Two genotypes from group 2 and one from group 3 unexpectedly retained high resistance when inoculated with isolate MP1580, although this isolate was virulent to *Rpi-Smira1* and *Rpi-phul* genes present in individuals of group 2 and 3, respectively. The other phenotypic traits of these recombinants remained in accordance with the MAS results for the presence of *R* genes. No other discrepancies were found in either group of genotypes.

Gene expression assay. Depending on the *P. infestans* isolate used and potato genotype tested, different reactions were observed during 5 days of DLA for gene expression analyses. In compatible interactions, the first symptoms (necrosis) covering approximately

Fig. 1. Box plots of the resistance of the four groups of genotypes (x-axis: 1 to 4) resulting from crossing of Sárpo Mira with the two clones Z-03.3817 and Z-03.3827. Genotypes possessed none (group 1), *Rpi-Smira1* only (group 2), *Rpi-phul* only (group 3), or both investigated resistance genes (group 4). Each group was tested with four *Phytophthora infestans* isolates, each displaying different virulence: **A**, MP324 was avirulent; **B**, MP324x and **C**, MP1498 were able to defeat the resistance conferred by single genes *Rpi-phul* and *Rpi-Smira1*, respectively; and **D**, MP1580 was virulent to the both resistance genes. Mean values are shown with the shape of a square, the box around it represents standard error, and the whiskers indicate standard deviation. Significant differences were calculated using Tukey's range test for unequal sample sizes with a significance level of 0.05. Genotypes of four tested groups do not differ significantly when marked with the same letter.

5% of a leaflet tissue were observed on the 3 dpi. Large lesions were produced on the leaflets of susceptible Craigs Royal inoculated with each of the four *P. infestans* isolates used. No symptoms developed on genotypes 04-IX-21 and Z11 with the *Rpi-phu1* gene when inoculated with isolates MP324 and MP1498, whereas isolates MP324x and MP1580 caused lesions covering nearly all of the leaflet surface on day 5 of the experiment. All of the *P. infestans* isolates triggered the HR in genotype Z5 with the *Rpi-phu1* and *Rpi-Smira1* genes. The plants of genotype 04-IX-21 spray inoculated with the isolate MP324 exhibited no symptoms of disease, whereas Craigs Royal control plants became infected.

Three leaflets of each genotype inoculated with each of the *P. infestans* isolates were used for RNA extraction and cDNA synthesis at each of the time points. The same cDNA was used in the qPCR experiments for expression analyses of the *Rpi-phu1* *R* gene and *Avr-vnt1*, *AvrSmira1*, and *Avr8* effectors. The three tested potato genotypes (04-IX-21, Z5, and Z11) revealed similar levels of *R* gene expression when inoculated with the MP324 and MP1498 isolates. No significant changes in *Rpi-phu1* transcript abundancies and no disease symptoms were observed after the inoculations until the last day of the experiment (Fig. 2). In compatible interactions at 2 to 3 dpi with the MP324x and MP1580 *P. infestans* isolates, a clear increase of *Rpi-phu1* relative expression was detected in the genotypes 04-IX-21 and Z11. The expression levels of the *R* gene continued to increase in these genotypes until day 5 of the experiment (Fig. 2). At this time point, the level of the *Rpi-phu1* transcript was significantly higher in the Z11 genotype than in other two genotypes inoculated with isolate MP324x. The transcription in genotype Z5 (*Rpi-phu1* + *Rpi-Smira1*) remained stable after inoculation with the MP324x and MP1580 isolates throughout the 5 days of the experiment. Similar transcript levels of the *Rpi-phu1* gene were observed in detached and undetached leaflets of the 04-IX-21 genotype inoculated with isolate MP324. In both cases, expression was maintained at a low and stable level, with no significant differences observed on at any of the six time points (Supplementary Fig. 1; Supplementary Table S1).

The relative expression levels of *Avr-vnt1*, *AvrSmira1*, and *Avr8* effectors were measured in the samples described above. However, measurements could not be done for combinations where no infection was observed: potato genotype Z5 inoculated with each of the

P. infestans isolates used and genotypes 04-IX-21 and Z11 inoculated with the isolates MP324 and MP1498. Measurements were not possible due to the absence of effector or *P. infestans* *Ej2α* reference gene transcripts or the high Cq value, exceeding the accepted level ($Cq < 40$). In infected leaflets, the relative expression of the effectors was measured on 5 days of the experiment. In the case of potato genotypes 04-IX-21 and Z11 inoculated with isolate MP1580, effector expression was detectable starting from day 2 or 3 (Fig. 3).

Among three effectors tested, *AvrSmira1* was expressed at the highest level in all experimental combinations. Statistically significant increase of the *AvrSmira1* transcripts was observed at 1 dpi in isolate MP1580, used for inoculation of Craigs Royal and genotype Z11 (Fig. 3B and F). The relative expression decreased within the next days of the experiment. Similar patterns were observed in the MP324x-inoculated 04-IX-21 and Z11 genotypes, although differences between expression at 1 dpi and other days were insignificant. Other combinations revealed stable expression patterns. The *Avr8* effector was expressed in all combinations at a low, stable level, with a slight increase of transcript abundance seen at 1 dpi and emphasized in genotype Z11 challenged with the isolate MP1580 (Fig. 3F). The transcript of the *Avr-vnt1* effector was absent until 3 dpi of genotypes 04-IX-21 and Z11 in the isolates MP324x and MP1580. The low level of expression was then present until the last day of the experiment. However, on susceptible Craigs Royal, the *Avr-vnt1* transcript was detectable on all days of the experiment, with the relative expression levels higher than those of *Avr8* in isolate MP324x (Fig. 3A).

As a validation of the effector assays, the transcript levels of the well-documented *Avr3a* effector (Armstrong et al. 2005) were investigated in the samples of Craigs Royal inoculated with MP324x and MP1580. Prior to that, we confirmed the presence of both avirulent and virulent alleles of the *Avr3a* gene in the DNA of these two isolates by using standard PCR with specific primers. We employed the same protocol for qualitative observations of the product abundancies in the cDNA of MP324x- and MP1580-inoculated Craigs Royal. No products were obtained with the *Avr3a*-F/-AR primer pair (avirulent allele) in either of samples. Using the *Avr3a*-F/-aR primer pair (virulent allele), we observed single products of expected size in MP324x samples and some additional unspecific products in MP1580 samples. As a consequence, qPCR

Fig. 2. A to D, Expression of the *Rpi-phu1* on three potato lines inoculated with four *Phytophthora infestans* isolates of different virulence. Relative expression (y-axis) was calculated using the ratios between quantification cycle (Cq) values of the target and the reference *α-tubulin* genes according to the $\Delta\Delta Cq$ method. Values on the x-axis indicate the day of the experiment. Data are average relative expression values of six biological replicates. The star indicates a sample that is significantly different from other samples shown in the figure according to Duncan's test, with the significance level of 0.05 calculated separately for A, B, C, and D.

was performed with the *Avr3a*-F/R pair, which exhibited higher specificity and yielded single-band products of expected size. The *Avr3a* gene product was present on each of 5 days of the experiment in both MP324x- and MP1580-inoculated Craigs Royal. Both sample sets exhibited an increase of the transcript level at 3 dpi, followed by a further decrease (Fig. 3A and B). No significant differences were observed between the expression of *Avr3a* and other investigated effectors.

Effector alleles. Sequencing of the *Avr-vnt1* and *AvrSmiral* effectors revealed three and four alleles, respectively, and their distribution among four *P. infestans* isolates used in this study (Table 2) can be found in Supplementary Table S3. These alleles were compared with the corresponding *Avr-vnt1* (PITG_16294) and *AvrSmiral* (PITG_07550) sequences from *P. infestans* reference strain T30-4. The comparison with the PITG_16294 sequence revealed one, four, and nine SNP in the 1, 2, and 3 *Avr-vnt1* alleles, leading to none, two, or six polymorphic amino acid residues (Supplementary Fig. S3). When the *AvrSmiral* 1, 2, 3, and 4 alleles were compared with the PITG_07550 reference sequence, 15, 13, 11, and 8 SNP were detected, of which 12, 8, 6, and 5, respectively, were nonsynonymous (Supplementary Fig. S5). The National Center for Biotechnology Information GenBank accession numbers of

the *Avr-vnt1* and *AvrSmiral* effector alleles can be found in Supplementary Table S3.

DISCUSSION

This study describes resistance profiles of potato breeding lines resulting from crosses between Sárpo Mira and *Rpi-phul* gene donors. A differential set of four *P. infestans* isolates and two molecular markers were used to dissect the resistance of progeny lines and revealed four groups diversified in their resistance. Two of these progeny lines, together with the additional 04-IX-21 genotype, were used in the *Rpi-phul* expression measurements after a pathogen challenge in compatible and incompatible interactions. The resulting patterns differed depending on the *P. infestans* isolate and potato genotype used. The transcript levels of the *Avr-vnt1*, *AvrSmiral*, and *Avr8* effectors investigated in the same experiment setup were also diversified. We showed that the expression patterns of both gene *Rpi-phul* and effectors can change within the host–pathogen interaction depending on the outcome of the interaction. Plants can enhance *R* gene expression when the infecting isolate is avoiding recognition, while the virulent pathogen can switch off expression of the effector when interacting with a plant able to recognize this effector.

Fig. 3. Relative expression of the *Avr-vnt1*, *AvrSmiral*, and *Avr8* effectors in the detached-leaflet assay on **A** and **B**, susceptible Craigs Royal and two potato genotypes carrying gene *Rpi-phul*—**C** and **D**, 04-IX-21 and **E** and **F**, Z11—inoculated with two *Phytophthora infestans* isolates (**A**, **C**, and **E**, isolate MP324x; and **B**, **D**, and **F**, isolate MP1580). Relative expression (y-axis) was calculated using the ratios between quantification cycle (Cq) values of the target and the reference elongation factor 2- α genes according to the $\Delta\Delta Cq$ method. Values on the x-axis indicate the day of the experiment. Stars indicate samples that are significantly different from other samples shown in the figure according to Tukey's range test for unequal sample sizes, with the significance level of 0.05 calculated separately for A to F. Due to a technical failure, the expression of the *AvrSmiral* effector could not be calculated at 1 day postinoculation in the MP1580 isolate used for inoculation of the 04-IX-21 genotype (**D**).

Resistance to late blight in Sárpo Mira × *Rpi-phul* gene donor progeny. Even though *R* genes such as *Rpi-phul* provide very high resistance, they may be overcome once a virulent isolate emerges, and such isolates have already been reported (Brylińska et al. 2016; Foster et al. 2009). Average scores of plants with the *Rpi-phul* gene were 9 when inoculated with isolates MP324 and MP1498 in our DLA. Similarly, the plants with gene *Rpi-Smiral* were highly resistant to MP324 and MP324x isolates. However, the resistance of individual genotypes, which were part of each of the four groups tested in the DLA, differed, which can be seen as the standard deviation values in Figure 1. Except for the presence of the two investigated genes, some lines could have other *R* genes or quantitative trait loci introgressed. The unexpected results were obtained for the plants with both *Rpi-phul* and *Rpi-Smiral* *R* genes. Isolate MP1580 successfully infected the plants without any and with each gene separately but was not able to overcome resistance in the group of plants with both genes present (Fig. 1D). This enhanced resistance could be explained by an interaction of two *R* genes or, as hypothesized above, the other *R* genes or quantitative loci stacked in Sárpo Mira and transferred to the progeny. As in our study, the same genotypes were used in DLA by Tomczyńska et al. (2014); however, in the latter study, the resistance profiles were tested with only two *P. infestans* isolates (MP324 and MP1353). The first isolate was also used in our study and the resistance profiles of the tested genotypes remained in agreement. Isolate MP1353 was able to overcome Sárpo Mira resistance but not the resistance conferred by the *Rpi-phul* gene, resembling isolate MP1498 used by us. Even though both isolates were of different races (Table 2) (Tomczyńska et al. 2014), the genotypes showed similar resistance patterns in both studies. Sequencing of the *AvrSmiral* effector of the isolates used in this study confirmed the earlier report of Rietman et al. (2012) that the two positively selected amino acids M¹⁵⁶ and R¹⁷⁰ do not correlate with virulence. Each of the *AvrSmiral* alleles found in our isolates carried Q¹⁷⁰ amino acid, whereas M¹⁵⁶ and L¹⁵⁶ amino acids were present in both virulent and avirulent isolates on plants with the *Rpi-Smiral* gene.

The effect of the *P. infestans* isolates' virulence on *Rpi-phul* transcript levels. The expression profiles of the *Rpi-phul* gene were examined in incompatible and compatible interactions in the three potato genotypes of different genetic backgrounds. A similar, constitutive expression was observed in all tested genotypes while inoculated with the *P. infestans* isolates MP324 and MP1498. The transcript level did not increase after the pathogen challenge and remained low until the last day of the experiment. This remains in agreement with the studies of Śliwka et al. (2013), where the *Rpi-phul* expression level was stable after pathogen contact in the 04-IX-21 clone also used in this study. The other two tetraploid breeding lines used in the studies of Śliwka et al. (2013) have also revealed stable expression. The *R3a* gene tested by Huang et al. (2005) also exhibited a constant expression level after interaction with an incompatible isolate. As shown by Bradeen et al. (2009), the level of *RB* transcript corresponded with late blight resistance. In our results, as well as those of Śliwka et al. (2013) and Huang et al. (2005), the initial amount of transcript was sufficient for fast *P. infestans* isolate recognition; therefore, the expression remained constant. The level of the *Rpi-phul* transcript in the genotype Z5, bearing both *Rpi-phul* and *Rpi-Smiral* genes, was stable on all days of the experiment, regardless of the isolate used, and resembled the expression patterns in tested genotypes with *Rpi-phul* inoculated with *P. infestans* isolates MP324 or MP1498 (Fig. 2), as well as in the MP324-inoculated tetraploid lines from the study of Śliwka et al. (2013).

A different type of response was observed when three tested potato genotypes were inoculated by MP324x and MP1580, the isolates that were able to overcome the resistance conferred by the *Rpi-phul* gene. In compatible interactions, the *R* gene exhibited increased transcript accumulation within the 5 days following inoculation in genotypes Z11 and 04-IX-21, resulting in expression of the experiment on day 5 that was 48 times higher than a basal expression (genotype Z11 inoculated with MP324x) (Fig. 2D). This pattern resembled the

increase of *RB* gene expression in the potato transgenic line carrying four copies of that gene (Kramer et al. 2009). The enhancement of the *Rpi-phul* gene was observed in three diploid lines in the study of Śliwka et al. (2013), where one line exhibited a significant increase on day 5 and the other two at 1 dpi. However, these diploid lines have shown no symptoms of an infection, in contrast to genotypes Z11 and 04-IX-21 of this study, which exhibited large lesion spots. Kramer et al. (2009) showed that all of the transgenic lines, regardless of the *RB* gene copy numbers, already exhibited enhanced transcription at 1 dpi when challenged with compatible *P. infestans* isolates. Increasing expression during infection was also reported for the NBS-LRR *QsRPe* gene, which provides resistance against *P. cinnamomi* in cork oak (Ebadzad and Cravador 2014). Although, in our experiment, the enhanced expression of the *Rpi-phul* gene did not result in resistance, it might be crucial for plants grown in a field where the infection pressure is usually lower. In our laboratory test, each suspension droplet used for inoculation contained approximately 1,500 sporangia, an amount which would be unlikely to appear simultaneously on a potato leaflet in a field. If the weather conditions are favorable, it takes a single *P. infestans* spore to develop the disease. Thus, it is possible that the *R* gene induction that we observed in our expression assay might prevent or significantly limit infection in the field.

To exclude the possibility that the observed *R* gene expression differences resulted from the stress caused by detaching the leaflets from plants, the transcript levels of the *Rpi-phul* gene from two separate sample sets were compared. Both sets consisted of 04-IX-21 leaflets inoculated with isolate MP324 but one contained the detached and the other undetached leaflets (i.e., collected from spray-inoculated plants). Comparison of the *Rpi-phul* transcript levels led to the conclusion that detaching the leaflets did not interfere with *R* gene expression (Supplementary Fig. S1; Table 1). Regardless of the procedure, the *Rpi-phul* gene exhibited low and stable expression, and no significant influence of the material source was revealed by the analysis of variance. It has been shown (Śliwka et al. 2013) that the higher the wild germplasm proportion, the higher the *Rpi-phul* basal expression observed. Similarly, a higher basal transcription of the *RB* gene has been reported in the wild potato species *S. bulbocastanum* than in any transgenic line containing one to four copies of that gene (Kramer et al. 2009). That leads to the conclusion that genetic background influences *R* gene expression, and this hypothesis was also supported in our study. The *Rpi-phul* gene basal transcription in genotype 04-IX-21 was higher than in Z11; these genotypes have had their wild germplasm proportions estimated at 3.12 and 1.56%, respectively.

Expression patterns of the *P. infestans* effectors *AvrSmiral* and *Avr8*. Measurements of effector expression were conducted on Craigs Royal and genotypes Z11 and 04-IX-21 inoculated with the isolates MP324x and MP1580. In each combination, the *AvrSmiral* effector revealed the highest transcript levels each day of the experiments. It belongs to the RXLR effector family; these effectors, in general, are known to be transcribed at the highest level at 2 dpi (Cooke et al. 2012; Haas et al. 2009). In our experiment, however, the *AvrSmiral* expression peaks were observed at 1 dpi, when isolate MP1580 was used to inoculate Craigs Royal and genotype Z11. Moreover, the *AvrSmiral* transcript level in the MP324x-inoculated genotype 04-IX-21 and Z11 resembled the above pattern, with its highest transcript abundance at 1 dpi, followed by a further decrease. Analogical patterns in the above combinations were also observed for *Avr8*; however, this effector is known to be expressed at the highest level already 16 h after inoculation (Rietman et al. 2012). Some of the effectors might exhibit expression peaks even at earlier time points. As reported by Chen et al. (2014), among the 17 candidate *P. cactorum* effectors with the RXLR domain, most had already peaked 1.5 h after the inoculation of the *Nicotiana benthamiana* host plant. Jupe et al. (2013) grouped the RXLR genes in *P. capsici* based on differential expression patterns during infection stages. Although some of the genes were induced on initial infection, the expression of others was shifted to the phase of transition from bio- to necrotrophy and yet

another group was specifically expressed in the late infection stages. The study of Jupe et al. (2013) showed that the RXLR effectors, depending on their function, might be upregulated at different time points, and some of them exhibited increased transcription at more than one infection stage. Whether the observed expression patterns in our study were of an isolate's nature or an effect of interaction with certain potato genotypes could not be resolved. Another explanation of the differences in the observed expression patterns could be isolate pretreatment. In other studies (Cooke et al. 2012; Haas et al. 2009), *P. infestans* isolates used for effector expression analyses were grown on artificial media, which could have affected the pathogen's physiology and gene expression. In our experiments, however, all of the isolates were propagated at least twice on leaves of the susceptible potato cultivars before the inoculation of the studied material. The expression of RXLR effectors was measured by Cooke et al. (2012) and Haas et al. (2009) in the mycelia grown on various media as well as during potato infection at 2, 3, 4, and 5 dpi. The average effector transcript level was, indeed, highest at 2 dpi yet no measurements were performed at 1 dpi. This means that our findings, revealing the highest expression of the investigated effectors already at 1 dpi in some cases, were not contradictory to earlier reports. To prove that our qPCR assays were valid, we analyzed the expression patterns of the *Avr3a* effector in MP324x- and MP1580-inoculated Craigs Royal. Our results were consistent with findings of Armstrong et al. (2005), who revealed the highest *Avr3a* transcript level at 3 dpi by measuring the expression at six time points between 12 and 72 h postinoculation. The same expression pattern with the *Avr3a* mRNA peaking at 3 dpi was observed in our study, which validated our approach (Fig. 3A and B). However, transcript levels can't be directly compared, because Armstrong et al. (2005) normalized the *Avr3a* relative expression to the expression in cDNA from cultured nonsporulating mycelium.

The observation that the transcript level of the *Avr8* effector in isolate MP1580 was 4.4 times higher at 1 compared with 2 dpi on genotype 04-IX-21 and 6.6 times higher on Z11 might suggest a possible influence of the genetic background of a host plant on effector expression patterns. This background could be, for example, some genetic factors inherited from Sárpo Mira in genotype Z11. However, this hypothesis could not be supported by comparing the transcript levels of the *AvrSmir1* effector due to a technical failure leading to no data at 1 dpi (Fig. 3D).

Expression patterns of the *Avr-vnt1* effector. Pel (2010), showed that there was no *Avr-vnt1* mRNA in any of 4 days of the experiment in which 'Bintje' plants were inoculated with a virulent isolate. Loss of an effector transcription was a strategy to avoid recognition, reported also for the *Avr3a* and *Avr1b-1* effectors in *P. sojae* by Qutob et al. (2009) and Cui et al. (2012). Similarly to the study of Pel (2010), in the compatible reactions in our experiment, no expression of the *Avr-vnt1* effector was observed on the first 2 days. A transcript appeared on further days, although at a low level. The reason for this discrepancy could emerge from different methods used, with a quantitative method used in our experiment and a qualitative method used by Pel (2010). The other possibility is a different potato genotype used in an experiment. Our results showed that the *Avr-vnt1* effector was expressed in isolates MP324x and MP1580 infecting susceptible Craigs Royal. In these combinations, the *Avr-vnt1* effector was detected on all 5 days of the experiment and was expressed in isolate MP324x on a higher level than the *Avr8* effector. The two *P. infestans* isolates both seemed to be able to switch off *Avr-vnt1* expression during interactions with the genotypes 04-IX-21 and Z11 bearing the *Rpi-phu1* gene. The *Avr-vnt1* expression shut down took place on the first 2 days of infection, preventing recognition by the *Rpi-phu1* gene and making these isolates virulent. The appearance of the *Avr-vnt1* effector on day 3 did not result in resistance. Thus, the expression patterns were clearly associated with a potato genotype used in the experiment. The interactions observed on 'Desiree' separately infected with two *P. infestans* isolates have revealed an expression pattern of *Avr-vnt1* characteristic for the RXLR effector family (Cooke et al. 2012). The *Avr-vnt1* effector transcript amount, which was highest at 2 dpi,

decreased in the next days of the experiment. However, a different microarray method than that in our study was used in the studies of Cooke et al. (2012) and Haas et al. (2009). The sequences of the *Avr-vnt1* gene from the MP324x and MP1580 isolates were compared with the PITG_16294 sequence from the *P. infestans* reference strain T30-4, used in the study of Cooke et al. (2012) and Haas et al. (2009). The sequences from virulent MP324x and the reference strain differed at a single, synonymous position and were identical to the sequences from avirulent strains MP324 and MP1498. The comparison of the reference sequence with the one from isolate MP1580 revealed nine different nucleotides leading to six polymorphic amino acid residues. Because the *Avr-vnt1* transcript levels in both MP324x and MP1580 were similar, we conclude that changes in the open reading frames could not be the sole reason that *Avr-vnt1* expression switched off at 1 and 2 dpi. Variation in the promoter regions, regulatory genes, or gene silencing could be responsible for the observed transcription patterns of *Avr-vnt1* and virulence in the tested isolates.

Conclusions. Unlike the studies of Kramer et al. (2009) and Śliwka et al. (2013), where *R* gene expression was examined in either compatible or incompatible interactions, we studied the *Rpi-phu1* transcript level with both types of *P. infestans* isolates. The expression pattern was shown to be different, depending on the virulence of an isolate. Results obtained using two virulent and two avirulent isolates showed a consistent pattern. We also confirmed the evasion strategy by disabling *Avr-vnt1* expression in the compatible reactions. However, in our study, the effector expression shut down took place only on resistant (*Rpi-phu1*) plants and only on the first 2 dpi. On susceptible Craigs Royal, the *Avr-vnt1* transcript was present on all of the tested days of the experiment. Hence, we conclude that the inactivation of the *Avr-vnt1* effector expression is not a simple genetic trait typical for a given *P. infestans* isolate but, rather, it is an ability to react to specific environmental factors, depending especially on potato genotype, *R* gene recognition, and HR activation. The quick resistance response in incompatible reactions already made the measurement of the *P. infestans* gene expression impossible on day 1 of experiment. The complexity of the potato host–pathogen interactions was emphasized in the results obtained in our DLA. Here, the potato genotypes of group 4 (*Rpi-phu1* and *Rpi-Smir1*) withstood the infection of MP1580, the *P. infestans* isolate virulent to both *R* genes separately. It will require further investigation to determine whether the enhanced resistance was a result of the interaction of *Rpi-phu1* and *Rpi-Smir1* or the presence of additional resistance loci.

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