Marker-assisted pyramiding of potato late blight resistance genes *Rpi-rzc1* and *Rpi-phu1* on di- and tetraploid levels

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Abstract Late blight is a disease with the biggest economic impact on potato cultivation worldwide. Pyramiding of the resistance genes originating from potato wild relatives is a breeding strategy that has a potential to produce potato cultivars durably resistant to late blight. Growing such cultivars would allow limiting the intensive chemical control of the disease. The goal of this work was to transfer the late blight resistance gene *Rpi-rzc1* from *Solanum ruiz-ceballosii* to the tetraploid level of cultivated potato and to pyramid it with the *Rpi-phu1* gene. We obtained two diploid and, through 4x-2x cross, a tetraploid potato population segregating for the *Rpi-rzc1* presence, as well as one diploid and one tetraploid population where both genes were introgressed. In total, 754 progeny clones were tested for resistance to late blight in detached leaflet assays. Pathogen isolates avirulent on plants with both genes and virulent on plants with the *Rpi-phu1* were used. The selection was assisted by two PCR markers flanking the *Rpi-rzc1* gene and a newly designed, highly specific intragenic marker indicating the *Rpi-phu1* gene presence. We obtained 26 diploid and 49 tetraploid potato clones with pyramid of both genes that should enhance

the durability and spectrum of their late blight resistance and that can be exploited in potato breeding. The specificity of the marker for the *Rpi-phu1* gene and the precision of the *Rpi-rzc1* mapping were improved in this work.

Keywords 13_A2 · Gene stacking · Interploid cross · *Phytophthora infestans* · *R* gene · *Solanum tuberosum*

Introduction

The cultivated potato, *Solanum tuberosum* L., and its wild relatives originate from South and Central America. Over 200 species of *Solanum* section *Petota*, to which *S. tuberosum* belongs, include species of various ploidy levels (diploids, tetraploids, hexaploids). Their taxonomy is affected by introgressions, interspecific hybridisations, sexual compatibility and phenotypic plasticity. They show diversity in plant height, tuber, leaf and flower colours and shapes (Ovchinnikova et al. 2011; Spooner et al. 2004). Wild potato relatives are adapted to plethora of environments that involve different precipitation regimes, day lengths, soils or latitudes and altitudes (Machida-Hirano 2015). They are used in potato breeding programs as sources of traits valuable from the breeder's and consumer's perspective, with resistance to biotic and abiotic stresses in particular.

Among the biotic stresses, the late blight caused by *Phytophthora infestans* (Mont.) de Bary is responsible for financial losses associated with yield decrease and cost of fungicides used to control the disease. Starting

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with a discovery of first 11 late blight resistance (*Rpi*) genes in *S. demissum* in 1950s, the number rose currently to over 60 identified genes from at least 25 *Solanum* species. All *Rpi* genes cloned so far contain nucleotide-binding site and leucine-rich repeats domains (Rodewald and Trognitz 2013). These domains were found in the reference potato genome in 755 genes distributed over 12 chromosomes in 92 gene clusters (Jupe et al. 2013). Because of the pathogen's ability for rapid adaptation, introduced *Rpi* genes may turn ineffective. As a promising strategy to extend the durability of resistance against late blight, gene stacking in a single potato cultivar was proposed. Introgressed *Rpi* genes should preferably originate from different gene clusters, thus differing in recognition specificity of pathogen gene products (Zhu et al. 2012). However, it was reported that the recognition spectrum of *Rpi* genes from different gene clusters might overlap, as it was proved for products of the *R2* and *Rpi-mcq1* genes recognising the same *P. infestans* protein (Aguilera-Galvez et al. 2018).

The late blight resistance genes were selected for pyramiding in this work because of the wide spectra of provided resistance, none or limited presence in cultivars so far, and locations on different potato chromosomes facilitating selection. The late blight *Rpi-rzc1* gene, originating from the diploid species *S. ruiz-ceballosii*, has been mapped to the chromosome X (Śliwka et al. 2012). The gene provides high level of resistance that has been rarely defeated by *P. infestans* isolates (Brylińska et al. 2016). The sequence of the *Rpi-rzc1* gene is not known. The sequence of the other *Rpi* gene used in this work, the *Rpi-phu1*, originating from diploid *S. phureja* and located at potato chromosome IX (Śliwka et al. 2006), is identical to the *Rpi-vnt1.1* gene from *S. venturii* (Foster et al. 2009). Also the *Rpi-phu1* gene provided high resistance against wide spectrum of *P. infestans* (Brylińska et al. 2016).

So far, no cultivar with the *Rpi-rzc1* gene is commercially available. Cultivars carrying the *Rpi-vnt1.1* / *Rpi-phu1* gene, like Polish cv. Gardena (registration in 2018) or Dutch cv. Alouette (registration in 2014), have already been released, which may exert selection pressure on pathogen population against that gene. Gene stacking might however slower down the process. Potato cultivars containing multiple *Rpi* genes maintain durable resistance of broader spectrum as reported for cvs. Bzura (*R2-like* + unidentified *Rpi* gene(s)), Sárpo Mira (*R3a*, *R3b*, *R4*, *R8* and *Rpi-Smira1*) or Mastenbroek's

differentials *R8* (*R3a*, *R3b*, *R4* and *R8*) and *R9* (*R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, *R9*) that possess at least two to seven known *Rpi* genes (Plich et al. 2015; Rietman et al. 2012; Kim et al. 2012). Rapid and efficient introduction of several *Rpi* genes into a single potato line was successfully applied by genetic engineering as done by Zhu et al. (2012), Jo et al. (2014) or most recently by Ghislain et al. (2019). The obtained genotypes exhibited broad-spectrum resistance to late blight and are being developed into registered potato cultivars.

The success of an *Rpi* gene introduction can be investigated by screening for resistance in phytopathological tests in laboratory or field conditions. However, such tests are laborious and time-consuming. Phenotypical tests can be replaced with molecular markers, which, in marker-assisted selection (MAS), speed up the process of selecting plants carrying the gene of interest.

The aim of this work was to transfer the late blight resistance gene *Rpi-rzc1* to the tetraploid level of cultivated potato and to pyramid it with the *Rpi-phu1* gene. For selection, besides phenotypical screening, we used molecular markers that flank the *Rpi-rzc1* gene and a marker derived from the *Rpi-phu1* gene sequence.

Materials and methods

Plant material

The plant material consisted of five unselected F1 potato progenies: three diploid (G2P-1, G2P-2 and G2P-3) and two tetraploid (4xG-1 and 4xG-2). The parents and numbers of individuals in the populations are presented in the Fig. 1. The crosses were performed between donors of the *Rpi-rzc1* gene and potato clones susceptible to late blight (populations G2P-1, G2P-3 and 4xG-1) or between donors of the *Rpi-rzc1* gene and donors of the *Rpi-phu1* gene (populations G2P-2 and 4xG-2). Pedigrees of the plant material are presented in Fig. 1a (diploids) and Fig. 1b (tetraploids). Origins of the genes *Rpi-rzc1* and *Rpi-phu1* were described by Śliwka et al. (2012) and Śliwka et al. (2006), respectively. Diploid late blight susceptible parents carried other useful traits: DG 97-2174 and DG 97-943 were suitable for chips production; DG 06-508 was resistant to pectinolytic bacteria and *Synchytrium endobioticum*.

Diploid progenies 2x-2x crossing programs were performed according to method described by Jakuczun and Wasilewicz-Flis (2001). Plants used as mother plants were grafted on tomato in order to extend the time of flowering. Flowers were emasculated at the bud stage 1 day before the pollination. Fresh pollen was collected from flowers of pollen parents and stored at about -20°C . The pollen fertility was assessed by the staining with lactofuchsin and observation of pollen grains at $\times 250$ magnification, as described by Wasilewicz-Flis and Jakuczun (2001a). The obtained fruits were allowed to mature and then the seeds were extracted. In the spring, the seeds were sown and the seedlings were grown in greenhouse in pots. After plant senescence, tubers of each genotype were harvested separately and stored at about $4-5^{\circ}\text{C}$ to the next planting season.

Tetraploid progenies To introduce the *Rpi-rzc1* gene into tetraploid potato germplasm, interploid ($4x-2x$) and then tetraploid ($4x-4x$) crosses were performed. In interploid crosses, a diploid potato hybrid clone DG 08-238, producing unreduced ($2n$) male gametes, was used as a pollen parent. Its ability to produce male $2n$ gametes was assessed based on the presence of big pollen grains, as described by Wasilewicz-Flis and Jakuczun (2001b). The tetraploid seed parent was a Polish cultivar Tajfun registered in 2004. A catalogue score of field late blight resistance of cv. Tajfun is 5 (in 1–9 scale, where 9 = the most resistant) but in our detached leaflet tests, this cultivar was susceptible (mean score 3.2, *P. infestans* isolate MP324). In order to extend the time of flowering, tetraploid mother plants were grafted on black nightshade plants (*Solanum nigrum* L.). Flowers were emasculated at the bud stage just before the pollination. The seeds were sown next spring and the seedlings were grown in greenhouse conditions. The ploidy level of these individuals was confirmed by counting of chloroplasts in guard cells, as described in Wasilewicz-Flis and Jakuczun (2001c). A $4x$ progeny clone G4-3-5 was chosen for next $4x-4x$ crosses based on phenotyping results and presence of DNA markers flanking the *Rpi-rzc1* gene.

Potato differentials With each late blight resistance test on dates shown in the Table 1, we performed simultaneously a virulence test against the Black's differential set (Black et al. 1953) obtained from Scottish Agricultural Science Agency, Edinburgh, UK, and following potato genotypes: cv. Bzura with the *R2-like* gene (Plich

et al. 2015), cv. Sárpo Mira with the *R3a*, *R3b*, *R4*, *Rpi-Smira1* and *Rpi-Smira2* genes (Rietman et al. 2012; Tomczyńska et al. 2014), cv. Biogold with the *Rpi-abpt* gene (Park et al. 2005), cv. Toluca with the *Rpi-blb2* gene (Zhu et al. 2015), breeding lines 04-IX-21 with the *Rpi-phu1* (Śliwka et al. 2013) and 99-10/36 with *Rpi-rzc1* genes (Śliwka et al. 2012); the isolate MP324x was additionally tested on cvs. Alouette (Armstrong et al. 2019) and Gardena known to contain *Rpi-phu1* / *Rpi-vnt1.1* gene (Potato Breeding Zamarte Ltd., Poland, personal communication).

Phenotyping

Detached leaflet assays (DLA) for late blight resistance were performed on fully expanded lateral leaflets collected from the 6-week-old plants as described by Brylińska and Śliwka (2017). Three *P. infestans* isolates were used (Table 2): MP324 used previously to identify the *Rpi-phu1* and *Rpi-rzc1* genes (Śliwka et al. 2006; Śliwka et al. 2012), a recent isolate MP1820 representing the 13_A2 (Blue-13) SSR genotype and MP324x virulent on plants with the *Rpi-phu1* gene (Stefańczyk et al. 2017). The assays were conducted in 2016–2019 according to the scheme shown in Table 1, one test each year with the exception of 2018 when two independent tests were done on different dates. Each test consisted of two replications with three leaflets of each potato individual per replication. The inoculum was prepared as described by Sobkowiak and Śliwka (2017) and adjusted to the concentration of 50 sporangia μl^{-1} . Leaflets inoculated with a pathogen sporangia suspension were incubated 1 day at 16°C /no light/abaxial side up, followed by 5 days at 16°C /1600 lx light/adaxial side-up conditions. Scoring was done after 6 days on a 1–9 scale with 1 meaning leaflet being completely diseased with intensive sporulation visible (Brylińska and Śliwka 2017; Online Resource 1).

Marker-assisted selection

DNA was isolated from young leaves of all potato progeny individuals and parental forms, frozen in liquid nitrogen, using the DNeasy Plant Mini kit (QIAGEN Polska Sp. z o. o., Poland) according to the producer's instructions. Three PCR markers were used for verification of the *Rpi-phu1* and *Rpi-rzc1* gene presence. The *phu1_2069* primers were designed based on the sequence of the *Rpi-vnt1.1* gene (NCBI GenBank:

Fig. 1 Pedigree of the material in which the *Rpi-rzc1* gene was introduced into cultivated potato breeding-pool and combined with *Rpi-phu1* gene, at diploid (a) and tetraploid (b) level. Unselected populations segregating for *Rpi-rzc1* or for *Rpi-rzc1* and *Rpi-phu1* genes are marked by rectangles with thick borders and number of individuals given. Grey rectangles mark diploid potato

clones DG 08–238 used as donor of *Rpi-rzc1* gene at diploid level and to transfer this gene into tetraploid potato breeding-pool. White rectangles with thin borders show potato breeding lines and cultivars. Resistant clones are marked by the names of resistance genes they were carrying; other clones were susceptible to late blight

FJ423044) that is identical to the *Rpi-phu1* gene (Foster et al. 2009). Primer pair (forward: 5'-CCAA ATTACTTGATCATGATT-3', reverse: 5'-TAGT ACCTGTGATATTCTCA-3') yielded a product that spanned 77% (2069 of 2680 bp) of the *Rpi-phu1* gene. PCR mixture (20 µL) contained sterile water, 1 µL of each 10 µM primer solution, 2 µL of 2 mM dNTPs,

4 µL of 5× high fidelity buffer and 0.4 U of Phusion DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, US). A touchdown PCR technique was used with initial denaturation for 180 s at 95 °C, followed by 7, 8 and 20 cycles of 95 °C for 30 s, 67/65/62 °C for 20 s and 72 °C for 45 s, ending with final elongation of 72 °C for 300 s. RenSeq1812 and RenSeq1910 primer sets were used for amplifying, respectively, 520 and 350 bp markers flanking the *Rpi-rzc1*, each in a distance of 0.4 cM (Brylińska et al. 2015). PCR mixture contained 0.4 µL of each 10 µM primer solution, 1 µL of 2 mM dNTPs, 2 µL of 10× PCR buffer and 1 U of *Taq* Polymerase (Genoplast Chemicals, Rokocin, Poland), filled up with water to 20 µL. Both markers were amplified using the following PCR program: 180 s at 95 °C, 30 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s, finished with 420 s at 72 °C. The products obtained with the RenSeq1812 and RenSeq1910 primers were digested with, respectively, *Bsa*II and *Hinf*I restriction endonucleases. For this purpose, 10 µL of PCR product was mixed with 3 U of restriction enzyme, 2 µL of 10× corresponding buffer

Table 1 Scheme of detached leaflet assays for resistance to *P. infestans* performed in years 2016–2019, using three isolates of the pathogen and five potato populations

Population	<i>P. infestans</i> isolate		
	MP324	MP1820	MP324x
G2P-1	2016, 2018 ^a	2017	–
G2P-2	2016, 2018 ^a	2017	2016, 2018 ^a , 2019
G2P-3	2016, 2018 ^a	2017	–
4xG-1	2019	–	–
4xG-2	2019	–	2019

^a In 2018, two independent detached leaflet assays were performed on different dates for each of the two *P. infestans* isolates

Table 2 *Phytophthora infestans* isolates used in detached leaflet assays to study the disease resistance of potato populations

Characteristic	<i>P. infestans</i> isolate		
	MP324	MP1820	MP324x
Virulence on:			
▪ Black's differentials	1.2.3.4.(5).6.7.(8).10.11 ^a	1.2.3.4.5.6.7.(8).10.11 ^a	1.(2).3.4.(6).7.(10).11 ^a
▪ Plants with <i>Rpi-rzc1</i>	Avirulent	Avirulent	Avirulent
▪ Plants with <i>Rpi-phu1</i>	Avirulent	Avirulent	Virulent
SSR genotype	Unique ^b	13_A2	Unique ^b
Mating type	A1	A2	A1
Metalaxyl resistance	Resistant	Resistant	Sensitive
Mitochondrial type	Ila	Ia	Ia
Isolation year	1997	2016	2012

^a Numbers indicate virulence towards the particular R1-R11 differentials (Black et al. 1953); numbers in brackets mean that the *P. infestans* isolate was virulent on this differential only in some tests, the missing numbers (e.g. 9) mean that the isolate was avirulent on given differential (e.g. R9); ^b a unique *P. infestans* SSR genotype

and filled up with sterile water to 20 µL. Mixtures were then incubated for 3 h at 55 °C (BsaJI) or 37 °C (HinfI). The PCR and digestion products were separated on 1.5% agarose gels stained with ethidium bromide and visualised using the UV light. Appearance of a 500 bp and/or 290 bp bands after digestion of the RenSeq1812 and RenSeq1910 PCR products indicated the presence of the *Rpi-rzc1* gene. The DNA samples of the potato tetraploid genotypes Z-03.3827 (Stefańczyk et al. 2017), 04-IX-21, cvs. Gardena and Alouette were used as the controls of the PCR assays carrying the *Rpi-phu1* gene, and of a diploid clone DG 99-10/36 (Śliwka et al. 2012) as a carrier of the *Rpi-rzc1* gene. DNAs of the late blight resistant cvs. Carolus and Kelly were used as negative controls in the *phu1_2069* marker amplification.

Statistical analyses

All the statistical analyses were performed using Statistica 10 software (Statsoft Inc. 2011). To test for the deviation between observed and expected segregation ratios, the chi-square (χ^2) test was applied. The correlations between the results of particular DLA done in different years and with the use of *P. infestans* isolates were assessed by calculation of Pearson's correlation coefficients. Analysis of variance and post-hoc Tukey's range test for unequal sample sizes were applied to study the effects of potato genotype and *P. infestans* isolate on late blight resistance scores.

Results

Crosses

The pollen fertility of DG 99-10/36, the donor of the *Rpi-rzc1* gene, was assessed as 50%, and no big pollen grains ($2n$ male gametes) were observed. This clone was crossed as pollen parent with two diploid clones DG 97-2174 and DG 97-943 (Fig. 1), and the efficiency of these pollinations was high. In the case of DG 97-2174 × DG 99-10/36 crosses, 36 flowers were pollinated and 22 berries were obtained. From these berries, we extracted in total 2594 seeds. In the case of DG 97-943 × DG 99-10/36 crosses, 22 flowers were pollinated and 9 berries were obtained. From these berries, 1112 seeds were extracted.

The *Rpi-rzc1*-containing diploid potato clone DG 08-238 was used as pollen parent in 2x-2x crosses as well as in 4x-2x crosses, to transfer the *Rpi-rzc1* gene from diploid to tetraploid potato gene-pool. The diploid level of DG 08-238 was confirmed, and the mean number of chloroplasts in guard cells was 8.6. The fertility of its pollen varied from 40 to 70%. The presence of big pollen grains ($2n$ gametes) in this clone was confirmed. From 282 flowers of diploid clone DG 06-508 pollinated with the DG 08-238 pollen, we obtained 43 berries and 1335 seeds. The efficiency of pollination in interpollid 4x-2x crosses was much lower. From 122 pollinated flowers of tetraploid cv. Tajfun, we obtained 19 berries and only 14 seeds, of which 12 plants emerged. The *Rpi-rzc1*-containing progeny clone G4-

3-5, obtained from 4x-2x cross, was tetraploid with the mean number of chloroplasts in guard cells 22.9. This clone was used as seed parent in 4x-4x crosses with tetraploid potato cultivar Felka Bona, and with *Rpi-phul*-containing tetraploid clone Bio-7. The effectiveness of these crosses was very high: 29 berries and 1280 seeds were obtained from cross G4-3-5 × Bio-7, while from cross G4-3-5 × Felka Bona, 47 berries and 4300 seeds were obtained. The ploidy level of 10 randomly selected clones from each of tetraploid progenies 4xG-1 and 4xG-2 was assessed, and all were tetraploid (range of mean of chloroplasts numbers: 20.8–23.0).

Genotyping

To detect the presence of the *Rpi-rzc1* gene, we obtained PCR products with the DNA of all 754 individual plants tested, using the RenSeq1812 and RenSeq1910 markers. After restriction digestion, the presence of the bands of approx. 500 and 290 bp, linked to the *Rpi-rzc1* gene, was scored for the RenSeq1812 and RenSeq1910 markers, respectively. There were four recombinants between the two markers, two in a diploid population G2P-1 and two in a tetraploid population 4xG-1. The numbers of RenSeq1910-positive and negative-individuals in each population and the recombinants are shown in Table 3.

In plants of populations G2P-2 and 4xG-2, the amplification of the *phul_2069* marker resulted in a single strong band of 2069 bp in size in the individuals with the *Rpi-phul* gene. As a positive control, the DNA of the 04-IX-21 potato line was used (Fig. 2). Another band of around 1900 bp can be additionally amplified using the marker: both bands were simultaneously obtained with the Z-03.3827 genotype (Stefańczyk et al. 2017) and with the cv. Alouette, while in cvs. Carolus and Kelly, the 1900 bp band was amplified only (Fig. 2). In total, there were 56 and 84 *phul_2069* marker positive individuals in populations G2P-2 and 4xG-2, respectively (Table 3).

Phenotyping

The virulence of the three *P. infestans* isolates in the DLA, taken as a consensus from historical (Stefańczyk et al. 2017; Śliwka et al. 2013; Tomczyńska et al. 2014) and the data obtained in this study, is summarised in the Table 2. None of the isolates was able to infect the 99-10/36 plants carrying the *Rpi-rzc1* gene. The plants with

the *Rpi-phul* gene: cvs. Gardena and Alouette, as well as potato lines 04-IX-21 and Z-03.3827, were infected only with the MP324x isolate and showed no symptoms of infection with other isolates used. The isolate MP324 was able to infect cv. Bzura, while cvs. Sárpo Mira, Toluca and Biogold remained moderately resistant. The isolate MP1820 exhibited similar virulence profile to MP324, except it completely infected cv. Biogold, but left cv. Sárpo Mira less affected by the disease.

All the individuals from the five potato populations were phenotyped in the DLA according to the scheme presented in the Table 1. Two *P. infestans* isolates used in the assays, MP324 and MP1820, were avirulent on plants with the *Rpi-phul* and *Rpi-rzc1* genes and of similar virulence profiles (Table 2). The resistance scores in DLA obtained with both isolates were strongly correlated in each of the populations surveyed (Pearson's correlation coefficients were 0.933, 0.977 and 0.958 at $p < 0.001$ for populations G2P-1, G2P-2 and G2P-3, respectively). The analysis of variance indicated no significant effect of the isolate on average resistance score (Online Resource 2). The resistance data obtained with the isolates MP324 and MP1820 were therefore pooled together for further analyses (Figs. 3 and 4).

Marker-assisted selection

In each potato population tested, the RenSeq1910 marker co-segregated with the resistance scored in DLA, while four individuals showed recombination between the RenSeq1812 marker and the gene (Table 3). For one recombinant from 4xG-1 population, a product with the RenSeq1812 marker was obtained, while no product was obtained with the RenSeq1910 marker, and DLA results indicated absence of the *Rpi-rzc1* gene. The other three recombinants (one from 4xG-1 and two from G2P-1 populations) gave no product with the RenSeq1812, whereas RenSeq1910 marker and phenotype tests indicated the *Rpi-rzc1* gene presence. The percentages of recombinants in the G2P-1 and 4xG-1 populations were estimated at 1.87% and 1.10%, respectively.

In two *Rpi* genes-stacking populations, where the *Rpi-phul* gene segregated, neither false negative nor false positive results were obtained with the *phul_2069* marker when we compared the phenotype and genotype of plants in three groups: without both *Rpi* genes, with the *Rpi-phul* or with the *Rpi-rzc1* only. In the groups with both genes, the presence of the *Rpi-*

Table 3 Segregation ratios of late blight resistance genes based on the PCR markers indicating the presence of the *Rpi-rzc1* (marker RenSeq1910) and *Rpi-phul* (marker phul_2069) genes,numbers of recombinants observed in each of the populations and results of the χ^2 test for the deviation of segregation ratios from the expectation

Population	Gene presence		<i>n</i>	Recombinants	χ^2 test for segregation		
	<i>Rpi-rzc1</i>	<i>Rpi-phul</i>			Ratio ^a	χ^2	<i>p</i>
G2P-1	–	n.a.	51	2 ^b	1:1	0.234	0.629
	+	n.a.	56				
G2P-2	–	–	50		1:1:1:1	11.714	0.008
	+	–	48				
	–	+	30				
	+	+	26				
G2P-3	–	n.a.	76		1:1	8.533	0.003
	+	n.a.	44				
4xG-1	–	n.a.	79	1 ^c 1 ^b	1:1	3.165	0.075
	+	n.a.	103				
4xG-2	–	–	44		1:1:1:1	8.602	0.035
	+	–	63				
	–	+	35				
	+	+	49				

^a The expected segregation ratios; ^b resistant, RenSeq1910-positive individuals without the RenSeq1812 marker; ^c susceptible, RenSeq1910-negative individuals with the RenSeq1812 marker; *n.a.* not applicable

phul gene was not verified phenotypically due to the lack of *P. infestans* isolate virulent on the *Rpi-rzc1* plants.

Based on the molecular markers and the DLA results, we diagnosed the presence of the *Rpi-rzc1* and *Rpi-phul* resistance genes in each of the examined potato population. The numbers of the individual plants containing both investigated *Rpi* genes (i.e. simultaneously positive for RenSeq1910, RenSeq1812 and phul_2069 markers)

were 26 and 49 in the diploid and tetraploid populations, respectively (Table 3).

The results presented in Figs. 3 and 4 are showing the resistance of plant material against *P. infestans* isolates avirulent (Fig. 3; Fig. 4a, c), or virulent (Fig. 4b, d), on plants with the *Rpi-phul* gene. The differences in resistance of plants with or without the *Rpi-rzc1* gene were significant in all populations and tests (Figs. 3 and 4). Plants with or without the *Rpi-phul* gene differed

Fig. 2 Band patterns obtained with the phul_2069 marker for selection of potato breeding lines and cultivars carrying the *Rpi-phul* gene (marked with +). An arrow indicates the product of amplification of the *Rpi-phul* gene fragment of 2069 bp

Fig. 3 Box plots of the resistance in three potato populations divided into groups with the genetic markers indicating the presence of the *Rpi-rzc1* late blight resistance gene (x-axis). The mean values of the resistance scores against the *P. infestans* isolates MP324 and MP1820 are shown as squares, the box 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. When marked with the same letter, groups do not differ significantly

significantly only in tests with the *P. infestans* isolates MP324 and MP1820 (Fig. 3; Fig. 4a, c). Individuals without the *Rpi-rzc1* gene that belonged to the G2P-1, G2P-3 and 4xG-1 populations did not differ significantly in resistance to late blight according to the Tukey's test and were scored at 2.4, 2.0 and 1.5 on average, respectively. Individuals from the same populations, but carrying the gene, had average resistance scores estimated at 8.8, 8.9 and 8.9 (Fig. 3), and the difference

between these groups was insignificant as well. In the G2P-2 and 4xG-2 populations, regardless of the isolate used, the infected plants without investigated resistance genes were evaluated in DLAs at 1.3–2.4 on average, while the diseased plants with the *Rpi-phu1* gene (tests with virulent *P. infestans* isolate MP324x) had their resistance estimated at 1.8–2.4, and no significant differences were observed between these groups. Similarly, the score of the healthy leaflets in each genotype/

Fig. 4 Box plots of the resistance in two potato populations divided into groups with the genetic markers indicating the presence of the *Rpi-rzc1* and *Rpi-phu1* late blight resistance genes (x-axis). **a, c** Mean results of tests with *P. infestans* isolates avirulent on *Rpi-phu1* plants, MP324 and MP1820. **b, d** Mean results of tests with *P. infestans* isolate MP324x, virulent on *Rpi-phu1*

plants. The mean values of the resistance against the *P. infestans* isolates are shown as squares; the box 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. When marked with the same letter, groups do not differ significantly

P. infestans isolate combination was 8.8–9.0 (Fig. 4) and did not differ significantly between the healthy individuals with the *Rpi-phu1*, with the *Rpi-rzc1* or with both genes present.

The results of the χ^2 test for 1:1 and 1:1:1:1 of one or two resistance genes segregation ratios, based on both DNA marker data and DLA results, are presented in Table 3. The assumed 1:1 segregation was confirmed for the G2P-1 and 4xG-1 potato populations, but rejected for G2P-3 in which a deficiency of individuals carrying the *Rpi-rzc1* gene was observed. According to the test, the numbers of individuals in four genotype groups did not fit to 1:1:1:1 ratio neither in the G2P-2 nor 4xG-2 populations (Table 3). In the G2P-2 potato population, an excessive amount of plants with no investigated genes, and with the *Rpi-rzc1* gene only, was observed. In the 4xG-2 population, there were too many individuals with the *Rpi-rzc1* and less than expected plants with the *Rpi-phu1* gene.

Discussion

Resistance genes stacking in a single cultivar should improve durability of the resistance, because it is less likely for a pathogen to simultaneously gain virulence towards a plant with several *R* genes. Gene stacking can be conducted by pyramiding *R* genes, different alleles of the same gene or even the same alleles in order to observe gene allele-dosage effect (Tan et al. 2010). In some plant species, cultivars with multiple *R* genes against various biotic stresses have shown durable resistance, e.g. in wheat against *Puccinia triticina* and *P. striiformis* for over 20 years each (Mallard et al. 2005; McCallum et al. 2016), in rice against *Magnaporthe oryzae* “throughout a century of cultivation” (Fukuoka et al. 2015) or in oilseed rape against *Leptosphaeria maculans* for at least 5 years (Brun et al. 2009).

This strategy is used also in potato breeding for some time now, as cultivars such as Pentland Dell from 1960s and Escort from 1980s, but also already the *R8* or *R9* Black’s differentials from 1950s, are known to contain multiple *Rpi* genes (Bormann et al. 2004; Tan et al. 2010). Some other promising genotypes, with multiple *Rpi* genes, are those obtained by Tan et al. (2010), Ghislain et al. (2019) or potato cvs. Bzura and Sárpo Mira (Plich et al. 2015; Rietman et al. 2012). The resistance of cvs. Pentland Dell and Escort turned

ineffective soon after registration of these cultivars. The durability of resistance of the *R8* and *R9* Black’s differentials has never been properly tested as they have never been cultivated on a large scale. These differentials still maintain high resistance against late blight (Brylińska et al. 2016), just as the late maturing cv. Sárpo Mira (Rietman et al. 2012) registered in 2002 and cultivated on moderate scale. Limited success of gene pyramids against potato late blight can be at least partially associated with *P. infestans* biology and partially with the choice of the *Rpi* genes used (Leesutthiphonchai et al. 2018). This pathogen is attributed with features that are known to accelerate adaptation and thus *R* gene pyramid defeat: its genome undergoes frequent modifications through mutations, the pathogen can reproduce sexually, its populations are large and not isolated which allows an increased gene flow (Stam and McDonald 2018). Ideally, *Rpi* genes used for stacking should not be previously present in any plant cultivar, so adapted isolates would not exist at all or exist in a pathogen population at low frequencies, but that’s rarely a case. To efficiently fight *P. infestans*, the strategy of using potato cultivars with stacked resistance genes should still be complemented with other management practices (Stam and McDonald 2018).

To respond to fast evolution of a pathogen, a breeder should have access to wide pool of *R* genes and tools for their fast introduction to breeding lines. Genetic engineering would extend possibilities of gene pyramiding as it allows transferring gene of interest from any source, regardless of crossing barriers, into a crop plant (Mekonnen et al. 2017). Recent advances in development of homozygous potato inbred lines are also promising and provide another approach for generating late blight resistant diploid hybrids. This strategy was used by Su et al. (2020) who obtained potato lines with pyramids of two *Rpi* genes in different combinations from four wild relatives: *S. avilesii*, *S. chacoense*, *S. tarijense* and *S. venturii*.

Stacking several resistance genes in a single genotype is challenging. Identifying plants with two or more genes solely by phenotyping can be impossible, if pathogen isolates able to overcome the resistance provided by each gene are not available. If DNA markers are available, whether linked to or based on a gene sequence, a resistance gene presence can be determined with ease. DNA markers solve also the difficulty of phenotypic tests being most often destructive for plants.

In this paper, the *Rpi-rzc1* gene was transferred from diploid to the tetraploid level and combined with the *Rpi-phu1* gene both in 2x and 4x forms. Individual plants from the obtained 2x and 4x populations were tested with *P. infestans* isolates, one of which was capable of infecting plants with the *Rpi-phu1* gene. We did not possess an isolate defeating *Rpi-rzc1*-conferred resistance, yet using a molecular marker based on the sequence of the *Rpi-phu1* gene asserted reliability of the genotyping approach. On the other hand, the sequence of the *Rpi-rzc1* gene is not known and genotyping was performed using two linked markers. As demonstrated in this study, they can be successfully used on both, di- and tetraploid levels. Pre-breeding is often performed on diploid level in potato, especially for introgression of wild germplasm and combining desired traits from different sources. Transfer of genes and DNA markers between ploidy levels may not be straightforward and requires validation, such as in this study, due to the effect of tetraploid cultivars' genetic background. The obtained breeding lines can be exploited in both di- and tetraploid breeding programs as well as studies on *P. infestans*–potato interactions.

Physical distance between markers RenSeq1910 (PGSC0003DMG400008595, chr10 52,694,086 bp) and RenSeq1812 (PGSC0003DMG400011045, chr10 53,377,529 bp) in the potato reference genomes DM1–3 is 683,983 bp (Brylińska et al. 2015). Genetic distance in the *S. ruiz-ceballosii* genome of these two flanking markers was previously estimated at 0.4 cM from the *Rpi-rzc1* gene (Brylińska et al. 2015), but this study indicated that RenSeq1910 is located closer to the *R* gene than the marker RenSeq1812. The distance of the RenSeq1812 marker to the *Rpi-rzc1* gene was estimated at 1.87 and 1.10 cM in the G2P-1 and 4xG-1 potato populations, respectively. The RenSeq1910 co-segregated with the *Rpi-rzc1* gene in all five tested progenies. In lack of intragenic markers, the linked ones should be closer than 5 cM genetic distance and preferably flanking the gene of interest (Collard and Mackill 2007).

Previous study reported no deviation from the 1:1 segregation ratio of the *Rpi-rzc1* gene (Śliwka et al. 2012). However, the results from studies on the *Rpi-phu1* gene were depending on the genetic background. In the diploid mapping population 97-30, two-thirds of plants were highly resistant (Śliwka et al. 2006). In three populations obtained later and with higher contribution of *S. tuberosum* genome, the gene segregated in the 1:1

ratio (Śliwka et al. 2010). In this study, three of five surveyed potato populations exhibited significant deviation from the expected 1:1 and 1:1:1:1 ratios as tested using the χ^2 test. This deviation might be caused by a distorted segregation, a phenomenon associated with genetic factors involved in reproduction and observed in plants, also extensively described in potato crosses (Manrique-Carpintero et al. 2016). Distorted segregation may be caused by genetic incompatibilities, but also by skewed sampling during population development, i.e. unaware preferential selection (Boopathi 2013).

In the previous work (Stefańczyk et al. 2017), we have used the *phu6* marker to indicate the presence of the *Rpi-phu1* gene in breeding lines derived from crosses between the gene donors and cv. Sárpo Mira. The *phu6* marker scores were in accordance with the phenotype in 84% cases (Stefańczyk et al. 2017). The disagreement was related to both false negative and false positive results but the latter were more frequent in total. The application of the *phu1_2069* marker in this work showed no discrepancy with phenotypes scored in DLA in the genotype groups, in which such comparison was possible. The new marker spanned a larger fragment of the *Rpi-phu1* gene and was more specific than the previous *phu6*.

In this work, we describe successfully developed di- and tetraploid potato populations and selection of 26 and 49 individual plants, respectively, that contain a pyramid of two broad-spectrum late blight resistance genes *Rpi-rzc1* and *Rpi-phu1*. We validated and improved specificity of the PCR markers used for selection that should accelerate and facilitate breeding, but only large-scale cultivation of the future potato varieties can demonstrate the durability of the resistance provided by this gene pyramid.

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Author's contributions ES optimised and applied PCR markers. JP performed some of the crosses and maintained the plant material and collected plant material for DNA extraction. SS maintained *P. infestans* cultures and prepared inocula for detached leaflet tests. MJ and JP performed detached leaflet tests. ES, JP and JS were involved in data analyses and manuscript writing. MJ, PSD and SS proofread the manuscript. JS contributed to designing the research.

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Availability of data and material All data are presented in the manuscript. *Phytophthora infestans* isolates are available from Młochów Potato Pathogen Collection and selected potato breeding lines from Młochów Potato Collection at Plant Breeding and Acclimatization Institute–National Research Institute, Poland.

Compliance with ethical standards

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

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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