Eur J Plant Pathol (2015) 143:193–198
DOI 10.1007/s10658-015-0663-2

Fine mapping of the *Rpi-rzc1* gene conferring broad-spectrum resistance to potato late blight

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Accepted: 15 April 2015 / Published online: 4 May 2015

Abstract The *Rpi-rzc1* gene originates from *Solanum ruiz-ceballosii*, a wild diploid relative of the cultivated potato. It provides high levels of resistance to late blight both in detached leaflet and in tuber slice tests. Here, we present evidence on the broad-spectrum of this resistance using detached leaflet assays and 509 diverse Polish *Phytophthora infestans* isolates of which only seven (1.4 %) were virulent on plants with the *Rpi-rzc1* gene. In a previous mapping study genetic distance between the *Rpi-rzc1* gene and locus *F* conferring violet flower colour was 3.4 cM, while on the other side the gene was flanked by marker T1521 located in the distance of 6.1 cM. Adding new RenSeq markers changed the map order and slightly decreased these distances. To further increase the precision of the genetic map we expanded the mapping population with another 240 individuals originating from the same cross. These individuals were assessed for resistance to *P. infestans* in detached leaflet tests and their flower colours were evaluated. Ten sequence-specific PCR markers were scored in the enlarged mapping population: two were derived directly from the NBS-LRR homologs from the potato genome sequence, two were identified by the Resistance Gene Enrichment Sequencing (RenSeq) approach, and the remaining six from other mapping studies. On the resulting map the *Rpi-rzc1* gene is flanked by markers located 0.4 cM from it. Narrowing down the chromosome sector containing the *Rpi-rzc1* gene to 1 cM improves the efficiency of marker-assisted selection and will be useful for studies aiming at cloning the gene.

Keywords Flower colour · Markers · *Phytophthora infestans* · *R* gene · *Solanum* · Virulence

Potato (*Solanum tuberosum L.*) is grown on ca. 20 million hectares worldwide and more than 300 million tons are harvested each year (Haverkort et al. 2013). These 300 million tons of staple food are endangered by an oomycete pathogen *Phytophthora infestans* (Mont.) de Bary causing potato late blight. The disease can result in severe yield losses or significantly increase the price of potatoes when controlled chemically. A sustainable solution of this problem could be breeding and growing potato cultivars resistant to *P. infestans*. The resistance can be found among numerous wild relatives of potato that bear both resistance (*R*) genes and Quantitative Trait Loci (QTL) affecting this trait. The advantages of exploiting the *R* genes are the high level of resistance provided and relative ease of their introduction to the cultivated gene pool either by crossing or by cisgenesis.

Electronic supplementary material The online version of this article (doi:10.1007/s10658-015-0663-2) contains supplementary material, which is available to authorized users.

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Recent list of functional *R* genes against potato late blight contains at least 68 genes from various *Solanum* species (Rodewald and Trognitz 2013). So far, 14 of them have been shown to belong to the same Coiled Coil – Nucleotide Binding – Leucine – Rich Repeat (CC-NB-LRR) gene family and further nine – to contain NB and LRR domains (Rodewald and Trognitz 2013). More late blight resistance genes are to be discovered since in a single doubled haploid genome DM (*S. tuberosum*) there are 755 NB-LRR genes annotated (Jupe et al. 2013) while in tomato (*S. lycopersicum*) 326 are found (Andolfo et al. 2014). How many of them are involved in interactions with *P. infestans* remains unknown but the above numbers of potential *R* genes grow when wild *Solanum* diversity, frequent polyploidy and heterozygosity are taken into account. Exploiting this resource in breeding potatoes resistant to *P. infestans* is until now limited. More *R* genes, effective and preferably broad-spectrum, are needed to be characterized and applied to defend against this damaging and fast-evolving pathogen.

One such gene is the *Rpi-rzc1* originating from *S. ruiz-ceballosii* (syn. *S. sparsipilum*) accession VIR 8664 (VIR 7370) (Śliwka et al. 2012). It was mapped to potato chromosome X using 114 diploid progeny of an interspecific cross between a late blight resistant *S. ruiz-ceballosii* clone 99–10/36 and a susceptible dihaploid of a *S. tuberosum* cultivar Balbina. The common linkage map of both parental clones included 1603 Diversity Array Technology (DArT) and 48 sequence-specific PCR markers. The *Rpi-rzc1* was shown to be linked to a potentially useful phenotypic marker that is violet flower colour, conferred by the locus *F* (van Eck et al. 1993, 1994). Genetic distance between *Rpi-rzc1* gene and locus *F* was 3.4 cM, while on the other side the gene was flanked by marker T1521 located in the distance of 6.1 cM. The gene provided high level of resistance in laboratory detached leaflet and tuber slice tests as well as in a field test (Śliwka et al. 2012). Later, two new RenSeq markers were scored in the same mapping population, which changed the map order and decreased the distances between the gene and closest markers to 3.4 and 3.7 cM (Jupe et al. 2013; Fig. 2a).

The goal of this study was to increase the precision of the genetic map of the *Rpi-rzc1* locus, providing potato breeders with more and better quality tools for marker-assisted introgression of the gene and supporting efforts towards its cloning. We expanded the mapping population with another 240 individuals originating from the same cross as described earlier (Śliwka et al. 2012). The 240 individuals were sown in 2012 and they were called 'population 12–4' to differentiate from the previous part of the progeny (Śliwka et al. 2012) that were sown in 2005 and named '05–18'. The individuals of the population 12–4 were assessed for resistance to *P. infestans* in detached leaflet tests in two years 2012 and 2013. Each year, three detached leaflets were tested per genotype in two replications. We used *P. infestans* isolate MP324 and a testing method described earlier (Śliwka et al. 2006, 2012). The resistance to late blight was scored in 1–9 scale where 9 meant the most resistant. The genotypes with mean resistance scores≥7 were considered resistant, i.e., containing the *Rpi-rzc1* gene. Segregation ratio of the resistant (N=121) and susceptible (N=119) individuals in the mapping population 12–4 did not deviate from the expected 1: 1 ratio, which was confirmed by the χ^2 test (χ^2=0.017, p=0.897). Flower colours of the individuals from the population 12–4 were scored visually as violet (N=119) or white (N=121). That trait also segregated 1: 1. There was clear linkage between resistance and flower colour with three resistant recombinants with white flowers and a susceptible one with violet flowers.

The donor of the *Rpi-rzc1* gene, *S. ruiz-ceballosii* clone 99–10/36 was evaluated for spectrum of the late blight resistance in detached leaflet tests using *P. infestans* isolates collected from different regions of Poland in years 2007–2013. Isolates from 2008 to 2009 (N=72) were included in a study on diversity of Polish *P. infestans* population that showed high genetic diversity and a potential for sexual reproduction of this pathogen in Poland (Chmielarz et al. 2014). In this sample of Polish *P. infestans* population, among 96 isolates, 66 different genotypes defined by 14 Simple Sequence Repeat (SSR) markers were found. These *P. infestans* isolates differed also in mating type, mitochondrial haplotype, metalaxyl resistance and virulence towards Black's differentials indicating that Polish population has not been dominated by a few clonal lineages (Chmielarz et al. 2014) which was still true in 2010–2013 (unpublished). Pure *P. infestans* cultures were obtained from leaves with single lesions according to the procedure described by Chmielarz et al. (2014). Then, they were propagated at least twice on susceptible potato tissues and tested on at least three detached leaflets of the clone 99–10/36. A *P. infestans* isolate was considered virulent when it produced sporulating lesions on the assayed leaflets and the mean resistance

score was<8. In total, 509 *P. infestans* isolates were tested, with numbers varying from 17 in 2013 to 140 in 2011 (Fig. 1). Virulent isolates were detected in samples from years 2008, 2009, 2011 to 2012 with low frequencies ranging from 0.008 (2012) to 0.070 (2009) (Fig. 1). An overall number of *P. infestans* isolates virulent on *Rpi-rzc1* plants was seven (1.4 %). Such low frequency of the virulent isolates in *P. infestans* population makes the *Rpi-rzc1* gene attractive for the potato breeding programs. Still, the virulent isolates are already present in Poland, even though plants with the *Rpi-rzc1* gene have never been cultivated in this area before. Taking into account that *P. infestans* is fast evolving and most likely propagating also generatively in this region (Chmielarz et al. 2014), there is a risk that with the growing acreage of *Rpi-rzc1* plants the frequency of virulent isolates will grow rapidly, rendering the gene ineffective. Therefore, the *Rpi-rzc1* gene must be released with caution, in a carefully chosen genetic background and in combination with other *R* gene (s). To enable faster introgression of the gene to a potato cultivar as well as successful pyramiding of resistance genes, we aimed at finding more closely linked and easy to use PCR markers.

Genomic DNA was extracted from 100 mg of fresh young leaves of 240 genotypes of 12–4 progeny and two parent plants grown in the greenhouse, using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the producer's instructions. Ten sequence-specific PCR markers were identified and scored in the mapping population 12–4: two were derived directly from the NBS-LRR homologs from the potato genome sequence, two were identified by the Resistance Gene Enrichment Sequencing (RenSeq) approach, and the remaining six from other mapping studies (Table 1). All sequence-specific markers listed in Table 1 were amplified in following conditions. The reaction mixture of 20 μl contained 2 μl of 10×PCR buffer, the four deoxynucleotides (0.1 mM each; Sigma-Aldrich, St. Louis, MO, USA), $MgCl_2$ (1.5 mM; GenoPlast Biochemicals, Poland), primers (0.2 μM; Sigma-Aldrich, St. Louis, MO, USA), *Taq* polymerase (0.05 U/μl; GenoPlast Biochemicals, Poland) and 10–30 ng of the template DNA. The PCR program was: 94 °C – 180 s; 40 cycles of: 94 °C – 30 s, annealing temperature given in Table 1–45 s, 72 °C – 90 s; 72 °C – 420 s. The reactions of PCR products with corresponding restriction endonucleases (Fermentas Life Sciences, Thermo Fischer Scientific Inc.), also listed in Table 1, were performed according to the manufacturer's protocols. PCR and digestion products were separated in a 1.0 or 1.5 % agarose gels stained with ethidium bromide and visualized under UV transillumination.

Marker data of four individuals from population 12–4 indicated that either these DNA samples were contaminated or that the plants originated from pollination with pollen that was not from clone 99–10/36. These individuals were excluded from further analyses. Population 05–18 contained some missing or uncertain phenotypes and genotypes. In order to avoid transferring these uncertainties to the fine mapping study, we analyzed population 12–4 separately. Linkage analyses were performed using JoinMap ® 4 (Van Ooijen 2006) with following settings: CP population type (creating only

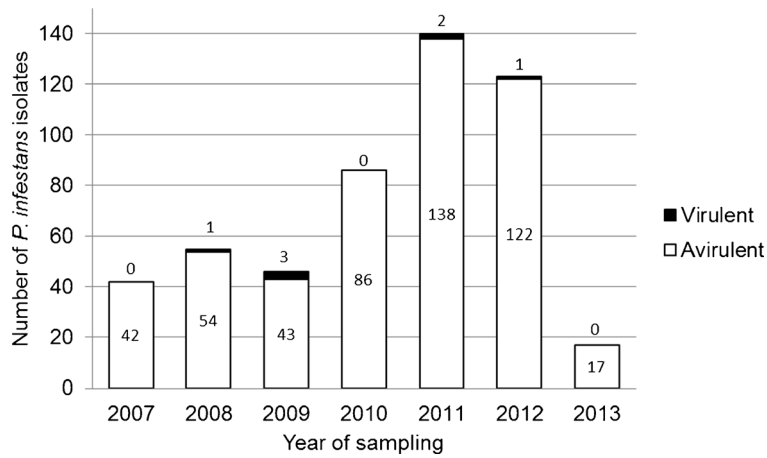

Fig. 1 Numbers of *P. infestans* isolates collected in Poland in years 2007–2013 that were virulent (numbers above the bars) or avirulent (numbers inside the bars) on detached leaflets of *S. ruiz-ceballosii* plants 99–10/36 (with the *Rpi-rzc1* gene)

Table 1 PCR markers used for fine mapping of the *Rpi-rzc1* gene

Name of the marker	Primer sequences 5′→3′	Ta (°C)	Product size (bp)	Restric-tion enzyme	Source
RenSeq1812	CCAACGCGTCCATCCATACACCTCC	55	520	*Bsa*JI	Jupe et al. 2013
	TGGTAAAGCTTACTGTGAATGATGTG				
RenSeq1910	CTTTTCCTATTGAAACATTGGAGCTA	55	350	*Hin*fI	Jupe et al. 2013
	GAAAGCTAAAGAGAAACAGAAACTTACG				
C2_At5g01350	TCCAATCTCAGCCATGGCTGG	55	2200	*Bsu*R1	SGN [b]
	TCTCTTTGATGTCCTCACTGCAAATC				
CT240	CCAAAGCCCAGGCTGTCAAG AGTCGGGTGTCACAATAA	55	900	*Hin*fI	Rauscher et al. 2006
RZC1	GGAGGCCAAAAGTTTGGAAG CGGCAGGCAATTAGGTTAGA	62	1300[a]	–	chr10: 53364766.. 53366765 [c]
RZC3	TGACTTATCGGAAGTTGGGACT GGCAAATTATACCGGAAGCA	58	1650	*Hin*fI	chr10: 56754525.. 56756306 [c]
TG63	CTGCATCAACTGGATATTCC	55	1200	*Tas*I	Park et al. 2009
	GTTGAGCAGTGCAATGTAC				
TG206	AAATCGAAAAGGGGCATACC	58	1000	*Tru*1I	Park et al. 2009
	TTGACATCCTCCAGCAGAAAC				
TG422	TGCATCTCTGTCCAAGCTCTATGC	55	440[a]	–	Park et al. 2009
	TGTGAGGCATTTTGATTCGCAC				
T1521	CAAGTATGGCAGGAACAAGTAA	55	850[a]	–	Śliwka et al. 2012
	ATAGACGACGAATTTCCAGCATA				

[a] Allele- specific

[b] SGN- www: SOL Genomics Network Database

[c] PGSC- www: Potato Genomics Resource Genome Browser Potato (*Solanum phureja* clone DM1-3 516R44) PGSC v4.03 Pseudomolecules

paternal linkage map for *S. ruiz-ceballosii* clone 99–10/36), independence LOD as a grouping parameter (linkages with LOD>3 were considered significant), regression mapping algorithm and Haldane's mapping function. Comparison of *S. ruiz-ceballosii* genetic maps based on populations 05–18 and 12–4 revealed several discrepancies (Fig. 2). They were likely caused by: i) missing phenotypes in population 05–18, ii) effect of DArT markers that were more prone to generating missing data and genotyping errors than PCR, iii) random effects of sampling that resulted in different sets of recombinants, and iv) genotyping mistakes. The fine map (Fig. 2b) was based on the population 12–4 where all individuals had the phenotype determined. Only PCR markers were used for construction of this map which allowed us to avoid the frequent missing od the data points characteristic for DArT markers. Therefore, the fine map should be considered not only more precise due to a greater number of individual but also of a better quality then the previous map based on population 05–18 (Śliwka et al. 2012).

Sequences (SGN) of the markers derived from literature or directly from SGN were aligned using BLAST to potato genome DM1-3 (PGR) in order to locate them in the potato genome (Supplement 1). Order of the markers on the genetic map of *S. ruiz-ceballosii* 99–10/36 was well corresponding with the order on DM1-3 physical map, with an exception of marker T1521. This discrepancy may be explained by the diversity of the tomato-derived sequence T1521 between DM1-3 and *S. ruiz-ceballosii* 99-10/36. The genetic and physical distances on the *S. ruiz-ceballosii* 99–10/36 map resemble ones of the DM1-3 genome in this region: while the distance of 5.6 Mb (from Chr10 51.1 Mb) is equal to 13 cM (0.43 Mb/cM) in DM1-3 genome (Sharma et al. 2013), markers CT240 (Chr10 51.4 Mb) and RZC3 (56.7 Mb) were located in a distance of 17.1 cM in *S. ruiz-ceballosii* (0.31 Mb/cM). On the genetic map

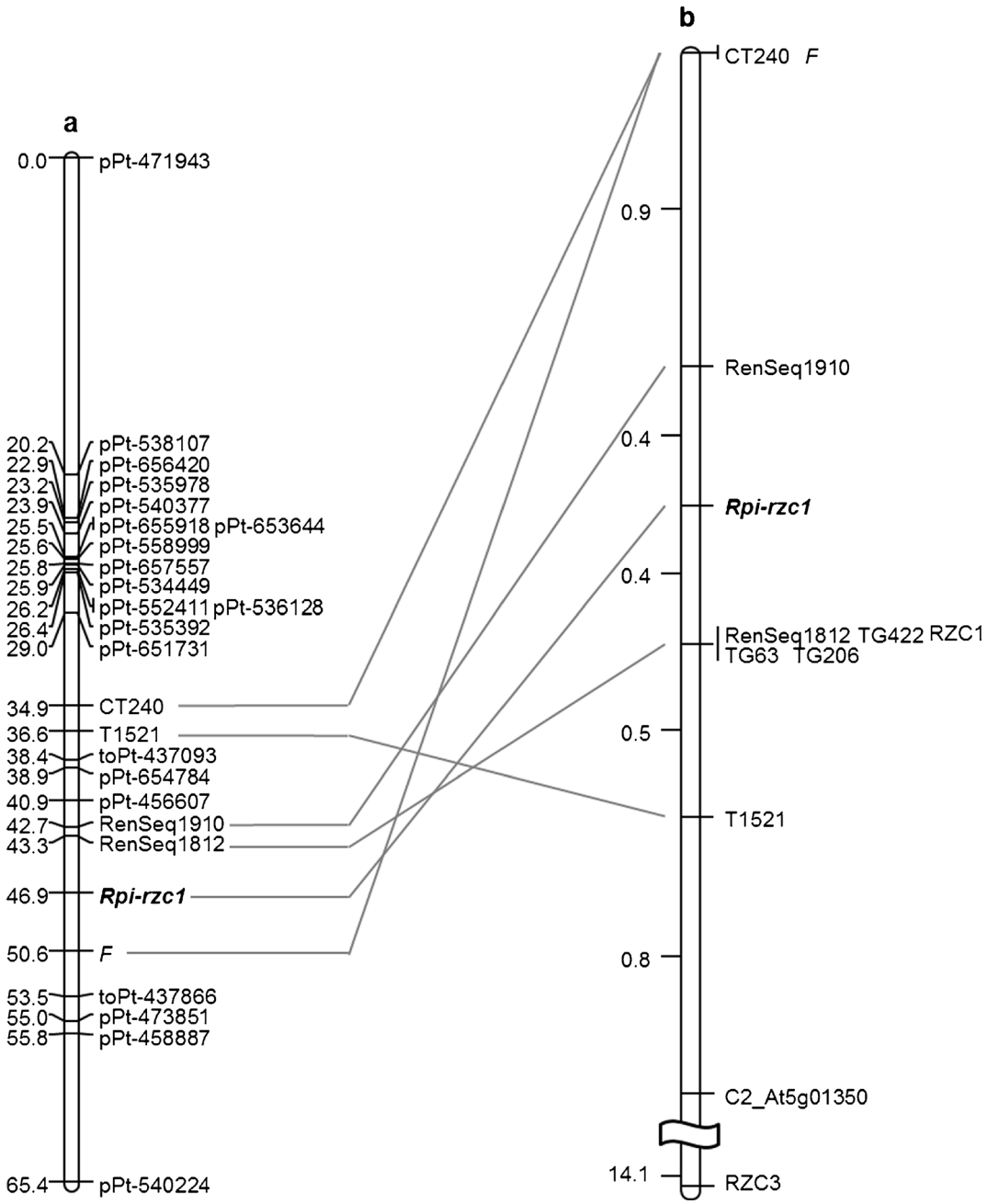

Fig. 2 Comparison of genetic maps of *S. ruiz-ceballosii* 99-10/36 chromosome X based on mapping populations: **a** 05–18, *N*=114 (Śliwka et al. 2012; Jupe et al. 2013) and **b** 12–4, *N*=236. Location of the *Rpi-rzc1* gene is marked in bold. On the left genetic distances in cM are given either as cumulative values (**a**) or intervals (**b**). Grey lines connect markers that are common to both maps

based on population 12–4 the *locus F* determining presence of anthocyanin pigment in flowers co-segregated with marker CT240 located 0.73 Mb from the genome position of the *locus F* reported in a tetraploid potato mapping study (Hackett et al. 2014). The *Rpi-rzc1* gene was flanked by markers located in a distance of 0.4 cM from it (Fig. 2b). Markers TG206, TG63, TG422, RZC1 and RenSeq1812 were redundant since no informative recombinants were found in population 12–4 (Fig. 2b). Co-segregation of DM1-3 NB-LRR-derived marker

RZC1 and the marker 1812 from RenSeq performed on *S. ruiz-ceballosii* confirms local conservation of the *R* gene clusters between those species.

Using a representative sample of current Polish population of *P. infestans*, the broad-spectrum of the resistance provided by the *Rpi-rzc1* gene was demonstrated. This resistance gene has not been exploited in potato cultivars so far and markers provided by this study can support the introgression of *Rpi-rzc1* to the different genetic backgrounds. Narrowing down the chromosome sector containing the *Rpi-rzc1* gene to less than 1 cM will improve the efficiency of marker-assisted selection and will be useful for future studies aiming at cloning the gene.

Acknowledgments This work has been financed by a statutory dotation 1-3-00-3-06 from the Polish Ministry of Science and Higher Education.

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SGN www: SOL Genomics Network Database http://solgenomics.net/.

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