

The effect of pyramiding *Phytophthora infestans* resistance genes $R_{Pi-mcd1}$ and R_{Pi-ber} in potato

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Abstract Despite efforts to control late blight in potatoes by introducing R_{pi} -genes from wild species into cultivated potato, there are still concerns regarding the durability and level of resistance. Pyramiding R_{pi} -genes can be a solution to increase both durability and level of resistance. In this study, two resistance genes, $R_{Pi-mcd1}$ and R_{Pi-ber} , introgressed from the wild tuber-bearing potato species *Solanum microdontum* and *S. berthaultii* were combined in a diploid *S. tuberosum* population. Individual genotypes from this population were classified after four groups, carrying no R_{pi} -gene, with only $R_{Pi-mcd1}$, with only R_{Pi-ber} , and a group with the pyramided $R_{Pi-mcd1}$ and R_{Pi-ber} by means of tightly linked molecular markers. The levels of resistance between the groups were compared in a field experiment in 2007. The group with $R_{Pi-mcd1}$ showed a significant delay to reach 50% infection of the leaf area of 3 days. The group with R_{Pi-ber} showed a delay of 3 weeks. The resistance level in the pyramid group suggested an additive effect of $R_{Pi-mcd1}$ with R_{Pi-ber} . This suggests that potato breeding can benefit from combining individual R_{pi} -genes, irrespective of the weak effect of $R_{Pi-mcd1}$ or the strong effect of R_{Pi-ber} .

Introduction

Late blight, caused by *Phytophthora infestans*, is a ruthless disease causing great losses in potato yield. One of the

solutions has been the use of chemicals. The International Potato Centre (CIP) estimates that about 1 billion dollars per year are spent on fungicides to control late blight in the US, Europe and developing countries (<http://gilb.cip.cgiar.org/what-is-late-blight/economic-impact/social-impact-and-economic-importance-of-late-blight/>). The loss in yield is nevertheless still considerable, and in addition, the environment suffers from the use of fungicides. In the 1900s, the first successful breeding efforts were undertaken to raise resistance levels of potato varieties by hybridization with wild potatoes (Salaman 1910). Eleven R_{pi} -genes ($R1-R11$), introgressed from *S. demissum*, were the first ones to be used in potato cultivars. But unfortunately soon after the introduction of many potato cultivars with *S. demissum* R -genes in the 1950s and 1960s, the resistance proved not to be durable. For a period of time, the prevailing strategy to achieve a durable solution against *P. infestans* was thought to be the use of horizontal (race-non-specific) resistance instead of R -genes in potato breeding (Colon et al. 1995; Landeo et al. 1995; Tan et al. 2008; Turkensteen 1993). Potato genotypes showing a hypersensitive response to *Phytophthora* races were discarded, because the non-durable R -genes would only blur the view on the level of horizontal resistance. Breeding for late blight resistance would require “ R_{pi} -gene free” germplasm (Turkensteen 1993). This strategy has not provided any improvement, mostly because of the inevitable correlation between resistance and late maturity (Simko 2002). Furthermore, it might be a valid assumption that residual effects of defeated R_{pi} -genes could be a factor in horizontal resistance (Stewart et al. 2003; Rauscher et al. 2008; Tan et al. 2008). These days the focus is back on R_{pi} -gene mediated resistance (Allefs et al. 2005). Since the discovery of the *S. demissum* R -genes, a wide range of wild *Solanum* species have been identified as potential sources for more R_{pi} -genes, e.g. species

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in the Dutch–German potato collection (Ross and Baerecke 1951; Van Soest et al. 1984). Eventually, the resistance genes R_{Pi-ber} from *S. berthaultii* (Ewing et al. 2000), $R_{Pi-mcd1}$ from *S. microdontum* (Tan et al. 2008) and four R_{Pi} -genes from *S. bulbocastanum*: $RB/R_{Pi-blb1}$, $R_{Pi-blb2}$, $R_{Pi-blb3}$ and $R_{Pi-abpt}$ (Park et al. 2005a, b; Song et al. 2003; van der Vossen et al. 2003, 2005) have been characterised. Furthermore R_{Pi} -genes similar to $R_{Pi-blb1}$ have also been found in *S. verucosum* (Liu and Halterman 2006) and in *S. stoloniferum* and *S. papilla* (Vleeshouwers et al. 2008). Currently, these R_{Pi} -genes are being deployed in breeding programs and one ($R_{Pi-blb2}$) is present in the commercial potato varieties “Toluca” and “Bionica” after more than 40 years of traditional crossing and breeding efforts (Hermesen 1966). However, not all of the R_{Pi} -genes render absolute levels of resistance in the practical field situation. Worse is the expected durability of these newly identified R_{Pi} -genes, as there is no reason to assume a longer durability of the new R_{Pi} -genes than of $R1-R11$, especially since these new R_{Pi} -genes have to oppose faster evolution rates of *P. infestans* with the introduction of the A2 mating type in the 1980s.

Pyramiding (major) R -genes can be one solution to improve on both durability and level of resistance (Nelson 1972). Pyramiding is the accumulation of (R)-genes into a single genotype or cultivar and can be achieved using major R_{Pi} -genes, defeated R_{Pi} -genes, different alleles of one gene, or the same alleles (allele-dosage). Although the potato cultivars such as Pentland Dell (1961) and Escort (1982) demonstrate that breeders were involved in pyramiding a long time ago, a surprisingly limited number of scientific reports have been published quantifying the effects of pyramided R -genes in different plant-pathosystems. Barloy et al. (2007) showed a higher level of resistance against cereal cyst nematodes in wheat when pyramiding resistance genes CreX and CreY. Several groups have reported on the pyramiding of bacterial blight resistance genes in rice and observed higher resistance levels and obtained additionally, a broader spectrum of resistance (Huang et al. 1997; Singh et al. 2001; Yoshimura et al. 1995; Zhang et al. 2006). When pyramiding multiple QTLs involved in resistance against barley stripe rust, Richardson et al. (2006) reported higher levels of resistance as achieved with individual QTLs. Whereas most studies show an additive effect of pyramiding two R -genes, the study of Sharma et al. (2004) did not. Marker-assisted pyramiding of the brown planthopper resistance genes *Bph1* and *Bph2* on rice chromosome 12 resulted in a resistance level of the pyramided line equivalent to that of the *Bph1*-single introgression line.

Slightly different are the examples of studies on the effect of allele-dosage. Toxopeus (1957) observed no additional *P. infestans* resistance in duplex R_3 or triplex R_3 genotypes compared to simplex R_3 genotypes. Likewise, no effect of allele dosage was observed in a study with H_1 , an

R -gene against *Globodera rostochiensis* in potato, where numbers of developed cysts were equal on genotypes containing simplex or multiplex copies of the H_1 gene (Brodie and Plaisted 1992).

In this paper, we describe a potato field trial in which the effect of pyramiding two *P. infestans* R -genes on late blight resistance is studied. The two R -genes R_{Pi-ber} (chromosome 10) and $R_{Pi-mcd1}$ (chromosome 4) used for pyramiding have been described in literature. $R_{Pi-mcd1}$ is described in Tan et al. (2008). To study the effect of pyramiding, it is necessary that both used R_{Pi} -genes do not provide absolute resistance. From our own experience with $R_{Pi-mcd1}$, an R -gene originating from *S. microdontum*, we knew that this R -gene gives a weak resistance to *P. infestans* in the field (Tan et al. 2008). Since both R -genes do not give absolute resistance, we deemed them suitable for studying putative quantitative differences in resistance when pyramiding these R -genes.

We developed a pyramiding population by crossing two diploid parents that were both heterozygous for one of the R_{Pi} -genes. In theory, the full-sib offspring can be divided into four groups, carrying no R_{Pi} -gene, with only $R_{Pi-mcd1}$, with only R_{Pi-ber} and a group with the pyramided $R_{Pi-mcd1}$ and R_{Pi-ber} . The advantage of making one pyramiding population instead of comparing cultivars with different and combined R_{Pi} -genes, is that the genetic background of the groups in one population is on average the same. The four groups cannot be differentiated by late blight assays, unless the different resistance spectra (if any) of the R_{Pi} -genes can be used. However, because the genetic position of both R -genes R_{Pi-ber} and $R_{Pi-mcd1}$ are known, we could make use of molecular markers flanking the R -genes. These molecular markers proved an accurate tool to genotype the pyramiding population and assign the genotypes into the four groups: (1) without R_{Pi} -genes, (2) with $R_{Pi-mcd1}$ present, (3) with R_{Pi-ber} present, and (4) containing both R_{Pi} -genes $R_{Pi-mcd1} + R_{Pi-ber}$.

Materials and methods

Plant material and *Phytophthora* resistance assays

Two segregating diploid populations were developed. The first population RH97-739 (see Fig. 1) was developed to verify the map position of R_{Pi-ber} and to develop flanking DNA markers. The second population RH03-424 (see Fig. 2) was developed to identify four groups of descendants without, with either or both R_{Pi} -genes.

The first population, which was used to characterise the R_{Pi-ber} gene used in this experiment was derived from *S. berthaultii* gene bank accession CGN 17823/PI265858. This gene might differ from the R_{Pi} -gene from the *S. berthaultii*

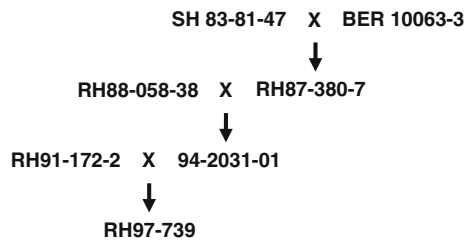


Fig. 1 Pedigree of RPi-ber mapping population RH97-739 used to develop flanking markers

gene bank accession PI473331 (Ewing et al. 2000 and Rauscher et al. 2006). To verify that the position of both R_{pi} -genes ($R_{pi-ber1}$ and R_{pi-ber}) is identical, a disease assay was performed with the interspecific mapping population RH97-739 (Fig. 1). Eighty-eight genotypes were tested in a field experiment in 2005, as well as in a detached leaf assay, whereas the population size for marker analysis was 79 descendants. The field assay was performed in two replications, in a randomised complete block design. Inoculation took place with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers et al. (1999). Infection was scored 18 and 30 days post inoculation (dpi). The genotypes were scored in a qualitative fashion at 30 dpi and were classified as either resistant when infection was $\leq 50\%$ of the average leaf area or susceptible when $\geq 95\%$ of the average leaf area was infected. The genotypes that fell in-between <50 and $>95\%$ were excluded from the qualitative genetic analysis. The detached leaf assay was performed with leaves collected from greenhouse plants and were tested in two replications as described by Vleeshouwers et al. (1999). The cultivar Bintje and parents RH91-172-2 and 94-2031-01 were included as control plants. Complex isolates IPO-82001 (race 1,2,3,4,5,6,7,10,11) and IPO-90128 (race 1,3,4,6,7,8,10,11) were used to determine leaf resistance. Inoculation was performed as described in Vleeshouwers et al. (1999) except that the concentration of the inoculum was adjusted to 5×10^5 zoospores/ml. In case of discrepancy between field and detached leaf assay results the field data were deemed most reliable.

The second population, also referred to as the pyramiding population RH03-424 ($n = 93$), descended from a cross between $R_{pi-mcd1}$ donor RH90-038-21, a BC1 of

S. microdontum accession BGRC 18302 (CGN21342), and R_{pi-ber} donor 94-2031-01, a BC1 of *S. berthaultii* accession BGRC 10063 (CGN 17823/PI265858). The pedigree of RH03-424 is presented in Fig. 2. The resistance gene $R_{pi-mcd1}$ is located on the short arm of chromosome 4 and gives a delay of infection to *P. infestans* (Tan et al. 2008).

In 2007, a field assay was performed on 93 individuals of pyramiding population RH03-424. The location of the field was the “Hoge Born”, in the area near Wageningen, NL on sandy soil. Experimental design was a randomised complete block design, in two replications. Per replication, 4 tubers per genotype were planted. Astarte, Bildtstar and Eersteling were used as standards. Tubers were planted on the 17th of April. Inoculation took place on the 26th of June with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers et al. (1999). Foliage infection levels were scored 15 times during 6.5 weeks. The percentage of diseased leaf area was recorded on a scale comprising 16 classes, corresponding to 0, 1, 2, 5, 10, 20, ..., 80, 90, 95, 99, 100 percent diseased leaf tissue. The four plants per genotype were scored as one experimental unit.

Maturity type was observed on a fungicide protected field experiment, on clay soil, in August of 2006 and 2007. Maturity type was scored approximately 130 days after planting on an ordinal scale ranging from 2 (green and vigorous; late to very late) to 6 (yellowing and plant is sagging) to 9 (dead; very early plant types). Four plants per genotype were treated as one experimental unit. Observations from 2006 and 2007 were averaged.

Maturity corrected resistance (MCR) was obtained essentially as described by Visker et al. (2003), where MCR is estimated from the initial resistance value using maturity as co-variate. MCR thus represents the difference between the observed resistance value and the resistance value as expected given its maturity. When resistance and maturity data from this population or from potato cultivars from the Dutch National List are plotted in a graph, the regression slope of their correlation is approximately -1 . This means that one unit gain on the maturity scale is decreasing late blight resistance with one unit (Visker 2005). Therefore, MCR values (or residuals) can be obtained easily by subtracting the maturity value from the resistance value. To display MCR values in a graph along

Fig. 2 Pedigree of pyramiding population RH03-424 which combines none, either or both the R_{pi} -genes from *Solanum microdontum* and *S. berthaultii*



with uncorrected resistance, the MCR values were increased with a constant value that equals the average maturity of the population. (MCR = foliage infection – maturity value + average maturity).

Genomic DNA extraction

Genomic DNA was isolated from young leaf material, harvested from young greenhouse grown plants, as described by van der Beek et al. (1992). Fresh tissue was ground at room temperature in STE-extraction buffer with steel balls using a Retsch machine (Retsch Inc., Haan, Germany). All volumes of the DNA extraction procedure were adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, USA).

DNA marker assays

The verification and mapping of the R_{pi-ber} gene was performed with CAPS markers CT214 (Rauscher et al. 2006) and TG63 and SCAR marker Q133. TG63F2/R2 primers were newly developed on TG63 DNA sequence of a genotype of *S. berthaultii* accession BGRC 10063 (CGN 17823/PI265858) amplified with TG63F1/R primers (Rauscher et al. 2006). Marker Q133, was newly developed using DNA sequence information (Genbank AF404451) of a Resistance Gene Analogue which maps to chromosome 10 of tomato (Pan et al. 2001). Genotyping of the resistance locus $R_{pi-mcd1}$ was performed with chromosome 4 specific CAPS marker TG339 (Tan et al. 2008). All primers were developed with Primer3 software (Rozen and Skaletsky 2000). Primers and PCR conditions are listed in Table 1. Subsequently, the flanking molecular markers were tested for usability on the parents of the pyramiding population because it might be possible that polymorphisms found in one population cannot be used in other populations. However, all markers were usable for genotyping RH03-424 with the restriction enzymes identified in the $R_{pi-mcd1}$ or R_{pi-ber} mapping population. The molecular markers allowed the pyramiding population to be divided into four groups:

(1) without R_{pi} -genes, (2) with $R_{pi-mcd1}$ present, (3) with R_{pi-ber} present, and (4) containing both R_{pi} -genes $R_{pi-mcd1}$ + R_{pi-ber} pyramided.

Linkage map construction of R_{pi-ber}

The marker order of chromosome 10 specific markers Q133, TG63, CT214 including R_{pi-ber} was determined by RECORD (van Os et al. 2005) and visually inspected by graphical genotyping the raw data in MS-Excel using the conditional formatting of cells conditional to marker genotype and linkage phase. Map distances were calculated based on the frequency of recombination between the markers, and in view of the short distances no adjustment for putative double cross-overs was made.

Statistical analysis

Analyses were performed with MS-Excel and comprised the chi-square test for goodness-of-fit and the analysis of group differences in resistance level in the pyramiding population using a 2-sided *t* test assuming unequal variances.

Results

Mapping of R_{pi-ber}

The response of the genotypes of the R_{pi-ber} mapping population RH97-793 in the field assay could be scored in a discrete fashion, where 49 genotypes could be scored as resistant and 37 as susceptible. The vast majority of the susceptible clones reached infection within 18 dpi, whereas the resistant clones were largely without symptoms. Only at 30 dpi did the first few resistant genotypes start to display infection (see Fig. 3). Two genotypes could not be scored unambiguously. The detached leaf assay showed the same segregation pattern as compared with the field assay, except for four differences, probably misclassifications due to mildew infection in the detached leaf assay. Therefore,

Table 1 Overview of the markers, primers and PCR conditions used in this study

Marker	Primer sequences 5' → 3'	Annealing temperature and extension time	RE digestion
CT214	F: GAACGCGAAAGAGTGCTGATAG R2: CCCGCTGCCTATGGAGAG T	Tm 60°C, 60 s	<i>DdeI</i>
TG63	F2: TCCAATTGCCAGACGAA R2: TAGAGAAGGCCCTTGTAAGTTT	Tm 55°C, 75 s	<i>BmeI390I</i>
TG339	F:GCTGAACGCTATGAGGAGATG R: TGAGGTTATCACGCAGAAGTTG	Tm 56°C, 90 s	<i>MnII</i>
Q133	F: ATCATCTCCTCAAAGAATCAAG R2: ATCTCCCCATTGACAACCAA	Tm 50°C, 30 s	

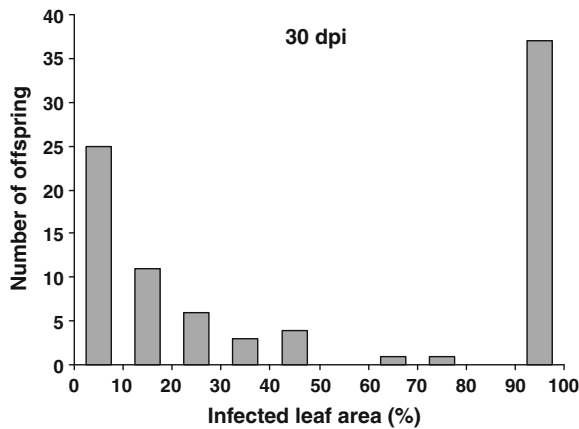
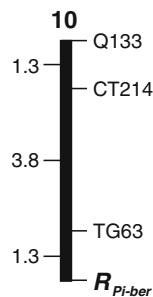


Fig. 3 Distribution of the percentage of infected leaf area at 30 days past infection of the offspring genotypes of the mapping population segregating for R_{Pi-ber} . From 17 until 30 dpi the U-shaped distribution allows unambiguous classification of resistant and susceptible segregants

Fig. 4 Genetic map of part of chromosome 10 of paternal resistant parent 94-2031-01 including the location of R_{Pi-ber}



subsequent analyses have been based on results of the field assay. Chromosome 10 specific marker loci Q133, CT214 and TG63 are closely linked with the locus for resistance, at distances of 6.4, 5.1 and 1.3 cM, respectively, north of R_{Pi-ber} . The order and distances of the markers towards R_{Pi-ber} was determined by five recombinants, and one singleton marker observation.

One of the five recombinants suggested the position of TG63 to be located north of R_{Pi-ber} . While taking this marker order, another descendant, with an ambiguous resistance phenotype then resulted in a singleton. Only the alternative marker order with TG63 south of R_{Pi-ber} would remove this friction, but would cause map friction in the recombinant with an unambiguous late blight phenotype. Therefore, the marker order as shown in Fig. 4 is the most plausible order as judged from the marker data.

Analysis of the pyramiding population RH03-424 with markers flanking $R_{Pi-mcd1}$ and R_{Pi-ber}

To identify which of either resistance genes are present in each offspring of the pyramiding population ($n = 93$), molecular markers flanking the R_{Pi-} genes were used. The pyramiding population RH03-424 could be differentiated

Table 2 Observed segregation of the descendants of the pyramiding population RH03-424 into four groups; without R_{Pi-} genes, with $R_{Pi-mcd1}$ present, with R_{Pi-ber} present, and containing both R_{Pi-} genes $R_{Pi-mcd1} + R_{Pi-ber}$ according to the flanking molecular markers on chromosome 4 and 10

	+ R_{Pi-ber}	- R_{Pi-ber}
+ $R_{Pi-mcd1}$	37	19
- $R_{Pi-mcd1}$	26	11

into four groups: (1) without R_{Pi-} genes, (2) with $R_{Pi-mcd1}$ present, (3) with R_{Pi-ber} present, and (4) containing both R_{Pi-} genes $R_{Pi-mcd1} + R_{Pi-ber}$ pyramided (Table 2). The observed segregation pattern into the four groups deviated significantly from the expected 1:1:1:1 ratio ($\chi^2 = 16.7$, $P < 0.001$). This deviation is largely due to an overrepresentation of resistant genotypes having the R_{Pi-ber} allele (63:30), whereas the $R_{Pi-mcd1}$ locus hardly deviates from a 1:1 segregating ratio 56:37 ($\chi^2 = 3.88$; $P = 0.05$).

Evaluation of the resistance level in the offspring of the pyramiding population

The results described below will first address the effect of the individual genes (a) $R_{Pi-mcd1}$ and (b) R_{Pi-ber} , respectively, (c) their interaction and (d) the effects after correction for maturity type.

R_{Pi-} gene free group versus $R_{Pi-mcd1}$ group

One week after inoculation, the R_{Pi-} gene free group had an average foliage infection of 66% (Fig. 5a). The group of descendants with the $R_{Pi-mcd1}$ allele showed infection as well, but to a lesser degree with 46% infection at this time point. The difference between the groups with and without the $R_{Pi-mcd1}$ allele was significant from 7 until 14 dpi (2-sided t test, $df = 24-27$, $P < 0.05$), with the largest effect at 9 dpi ($P = 0.0027$). The difference was no longer significant at 17 dpi and later time points, when both groups with or without $R_{Pi-mcd1}$ approached 100% infection.

Resistance level of R_{Pi-ber}

The difference in infection level between the groups with or without the R_{Pi-ber} allele is much larger compared to the effect caused by the $R_{Pi-mcd1}$ allele (Fig. 5a). When the foliage of plants from the R_{Pi-} gene free group is more than 90% infected, the R_{Pi-ber} bearing groups remain almost free from *Phytophthora* symptoms, with an average degree of infection below 5%. This absence of *Phytophthora* infection as the effect of the R_{Pi-ber} allele, remains until 24 dpi, a full 3 weeks longer than the R_{Pi-} gene free group, as well as the $R_{Pi-mcd1}$ group. R_{Pi-ber} does not, however, provide absolute

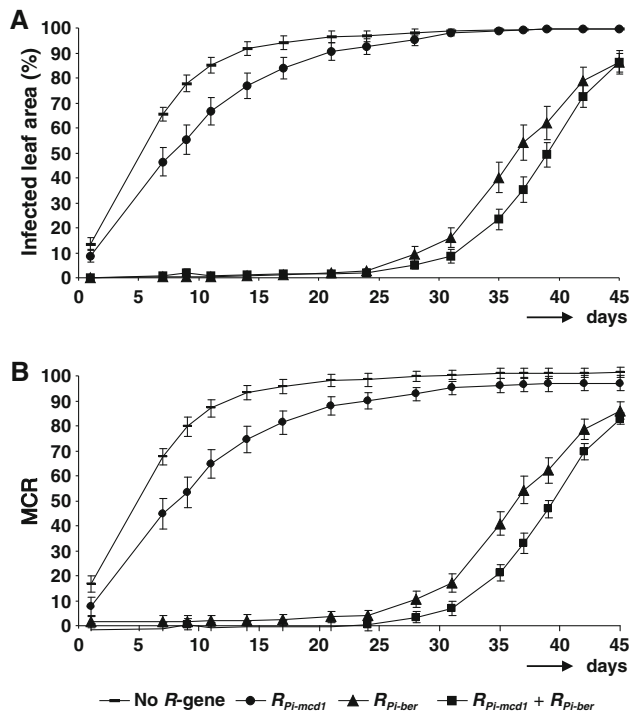


Fig. 5 Late blight disease progress curve showing the proportion of leaf area infected across 45 days post infection (dpi) of the four groups of pyramiding population RH03-424; without R_{pi} -genes, with $R_{Pi-mcd1}$ present, with R_{Pi-ber} present, and containing both R_{pi} -genes $R_{Pi-mcd1} + R_{Pi-ber}$. Error bars indicate the standard error of the mean. **a** Uncorrected disease progress using values as observed in the field. **b** Disease progress values using maturity corrected resistance (MCR) values

resistance. At 24 dpi, these plants also start to show late blight infection. In this experiment, the genotypes with R_{Pi-ber} passed the level of 50% infection on average at 36 dpi, 6 times later than the R_{pi} -gene free genotypes and 4.5 times later than genotypes with $R_{Pi-mcd1}$.

Effect of pyramiding $R_{Pi-mcd1} + R_{Pi-ber}$

From the first observation date, the presence of the R_{Pi-ber} allele had a more noticeable effect as compared to the presence of the $R_{Pi-mcd1}$ allele. The effect of pyramiding of the two resistance genes can only be observed when the plants having the R_{Pi-ber} allele (with or without $R_{Pi-mcd1}$) also showed infection. A significant contribution (2-sided t test, $df = 45-48$, $P < 0.05$) of the $R_{Pi-mcd1}$ allele in plants with R_{Pi-ber} was observed from 35 dpi and remains significant till 37 dpi, with the largest effect observed at day 35 ($p = 0.026$) (Fig. 5a). Considering that the genetic background of the R_{Pi-ber} group and the pyramided groups are on average the same, we conclude that the difference in resistance level was due to the additive effect of the $R_{Pi-mcd1}$ allele. The magnitude of this effect is comparable with the difference observed between the R_{pi} -gene free group and

the $R_{Pi-mcd1}$ group: 50% infection was achieved 3 days later in the pyramided group than in the group containing only the R_{Pi-ber} allele.

The effect of plant maturity on late blight infection

Within each group, there is considerable variation in the level of infection per genotype, where some genotypes containing the R_{Pi-ber} allele may have a level of infection which is lower than some genotypes in the pyramided group. The infection of both the R_{Pi-ber} groups begins at 24 dpi, which is 3 months after planting, long enough for first early cultivars to end their life cycle. Maturity types in the pyramiding population varied between 4 and 9 with a mean of 6.9 on a total scale of 1 to 9. Differences in maturity type could potentially influence the level of late blight resistance. The strong correlation between *P. infestans* foliage resistance and late plant maturity resulted in the use of maturity corrected resistance (MCR) (Bormann et al. 2004; Visker et al. 2004). Therefore, we re-analysed our late blight resistance observations using a maturity corrected dataset, where late blight resistance is compensated for the effect of maturity. MCR was calculated in this study by subtracting maturity values from leaf area infection values, but adding up the average value for maturity of the population, leaving the residuals. This is a simple and valid approach, because analysis of variance components showed that the variance contributed by maturity is of the same magnitude as the variance contributed by the R_{pi} -gene composition.

In Fig. 6, the effect is illustrated of this correction for maturity on late blight infection at 35 dpi. When comparing the maturity uncorrected (Fig. 6a) versus the maturity corrected (Fig. 6b) analysis, it becomes apparent that the correction for maturity slightly improves the difference in resistance levels between the R_{Pi-ber} group and the $R_{Pi-ber} + R_{Pi-mcd1}$ group. In addition, the correction for maturity

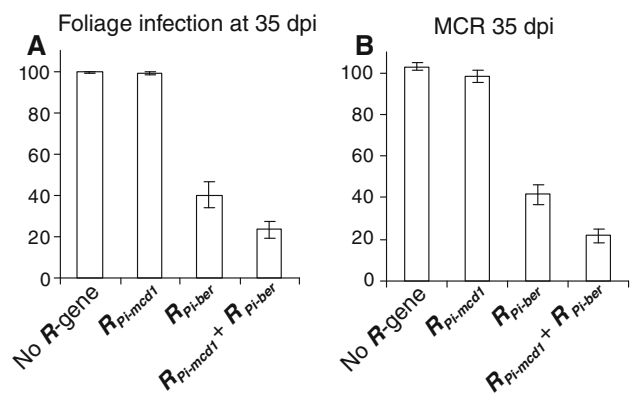


Fig. 6 Foliage infection (in %) of offspring genotypes classified according to R_{pi} -genes **(a)** Group averages of foliage infection at 35 dpi. **(b)** Group averages of maturity corrected resistance (MCR) at 35 dpi. Error bars indicate the standard error of the mean

reduced the standard error of the group mean. Removing the heterogeneity in maturity between plants within the four groups thus allowed a more accurate assessment of late blight resistance differences due to R_{pi} -gene composition. The correction for maturity also confirmed that quantitative variation in the observed level of infection was not only caused by R_{pi} -genes but in part caused by maturity. Lastly, the maturity corrected analysis showed that the effect of pyramiding remains statistically significant for a much longer period, from 31 until 39 dpi (9 days) instead of 3 days (2-sided t test, $df = 47-50$, $P < 0.05$), with the largest effect at 35 dpi ($P = 0.0014$). In Fig. 5b, the progress in time of the maturity corrected foliage infection for the four groups is illustrated.

Discussion

The individual R_{pi} -genes used in this study have been described before in the literature. The phenotypic effects of resistance based on $R_{pi-mcd1}$ are described in Tan et al. (2008). It was then estimated that $R_{pi-mcd1}$ causes a delay of infection of about 1–2 weeks. In this experiment though, genotypes with $R_{pi-mcd1}$ pass the level of 50% infection on average 3 days later than R_{pi} -gene free genotypes. The shorter delay of infection by $R_{pi-mcd1}$ in this experiment is likely caused by the relatively cold and humid weather favouring rapid late blight development in the summer of 2007.

Rauscher et al. (2006) mapped R_{pi-ber} 5.1 cM south of marker CT214 and 0.6 cM north of marker TG63. The resistance we observed in this study mapped to chromosome 10, close to marker TG63, but 1.3 cM south of marker TG63. Although the position of marker TG63 relative to R_{pi-ber} differs in this study with the marker order shown by Rauscher et al., we do not wish to draw strong conclusions on only one recombination event. Therefore, we cannot present conclusive evidence that this resistance is a different locus to the previously reported R_{pi-ber} . The difference in position of marker TG63 could be attributed to false positive/negative marker or phenotypic scoring which can make a large impact when dealing with a small population and a small number of markers. An alternative explanation could be that marker TG63 is located inside (and thus flanked by) a large cluster of R_{pi} -gene homologues. The use of one accession or another accession with different functional members of the cluster could lead to alternative mapping orders. R_{pi-ber} , originating from *S. berthaultii*, was reported as a new R_{pi} -gene by Ewing et al. (2000) and Rauscher et al. (2006). According to information on internet by Dr. J. Jones, there are two R_{pi} -genes located on chromosome 10 of *S. berthaultii* named $R_{pi-ber1}$ and $R_{pi-ber2}$ (http://www.ayeaye.tsl.ac.uk/index.php?option=com_content

[&task=view&id=98&Itemid=59](#)). They are both roughly in the same position as the previously identified R_{pi-ber} (Ewing et al. 2000; Rauscher et al. 2006). $R_{pi-ber1}$ may be the same gene as R_{pi-ber} . Because we were uncertain if the *S. berthaultii* resistance from our accession CGN 17823/PI265858) was the same locus as the previously identified R_{pi-ber} , we confirmed its map position using data from a field experiment. The data of this field experiment also confirmed that the resistance conferred by R_{pi-ber} is not absolute, but can be described as strong in effect.

The objective of this research was to pyramid two functional R_{pi} -genes originating from wild potato species in a *S. tuberosum* background and to compare the resistance level of those pyramided R_{pi} -genes with the level of the single R_{pi} -genes. We combined $R_{pi-mcd1}$ with R_{pi-ber} . Both R_{pi} -genes confer incomplete resistance to *P. infestans* but greatly differ in their effect. The $R_{pi-mcd1}$ gene offers a small but significant reduction of the infection, lasting only for a short period while the R_{pi-ber} resistance gene offers a strong effect. It took 36 days until 50% of the average leaf area was infected in plants that carry the R_{pi-ber} resistance gene. In the field trial, the plants with the R_{pi-ber} resistance gene did not confer immunity during the entire growth cycle. The failure of the resistance by the end of the growing cycle is an annually observed phenomenon, known from other trials and years using this genetic material (data not shown). This is best explained by the physiological aging of the plant. Pyramiding of $R_{pi-mcd1}$ and R_{pi-ber} resulted in a higher level of resistance. This is not an obvious conclusion that should have been expected before starting this experiment. In view of the large effect of the R_{pi-ber} gene until late in the growing season, and in view of the short delay of infection at the beginning of the infection period we could also have expected that effect of the R_{pi-ber} gene would entirely surpass the modest effect of the $R_{pi-mcd1}$ gene. The resistance level in the pyramid group showed an additive effect of $R_{pi-mcd1}$ with R_{pi-ber} without clear evidence for a ‘law of diminishing returns’.

In our experiment, we used a large population of full sibs. This sibling structure would on average nullify the interaction between a specific genetic background and the R_{pi} -genes. One example of such an interaction is the influence of maturity type on resistance level, irrespective of R_{pi} -gene composition. This experimental design proved to be effective, because it allowed us to compensate for maturity effects. In our experiment, the magnitude of the effect caused by the individual R_{pi} -genes was slightly obscured by variation in maturity. When resistance levels were corrected for maturity type, the difference between pyramided and the single R_{pi-ber} resistance levels stayed significant for a longer period of time: 9 days instead of 3 days.

The pyramiding of R_{pi} -genes is a valid strategy if the anticipated benefits exceed the breeding efforts. It is

debatable if an infection delay of a couple of days is valuable. In our pyramided group, the resistance level of R_{Pi-ber} was complemented by the resistance level of $R_{Pi-mcd1}$. $R_{Pi-mcd1}$ is known to be effective for a limited period. Therefore, improvement of resistance levels would be more economic when using R_{Pi} -genes with stronger effects. Irrespective of the complexities of natural infection with late blight, our findings indicate that combining the R_{Pi} -genes $R_{Pi-mcd1}$ and R_{Pi-ber} in potato is useful. In view of the additivity of the effects of the $R_{Pi-mcd1}$ and R_{Pi-ber} genes, and the lack of evidence for the ‘law of diminishing returns’, we recommend the development and analysis of potato clones with additionally pyramided R_{Pi} -genes to investigate the hypothesis that resistance levels comparable to non-host resistance might be feasible (Heath 2001; Jeuken and Lindhout 2002).

More important than economic considerations, the strategy of pyramiding R_{Pi} -genes is valued because of epidemiological and evolutionary considerations. Besides raising the level of resistance, pyramiding R_{Pi} -genes might contribute to the durability of resistance. The pathogen would need double or multiple mutations to overcome the resistance. The durability of an R_{Pi} -gene can be perceived as the evolutionary potential (mutation and recombination) of the plant pathogen to avoid R_{Pi} -gene recognition. In that case R_{Pi} -gene durability is based on the evolution of effector molecules and the indispensability of their current function. But when discussing durability of single or pyramided R_{Pi} -genes, one should be aware that durability can not be predicted in advance, but can only be reflected upon by historical evaluation. To ensure a maximum lifespan of an R_{Pi} -gene though, it is important that single R_{Pi} -gene and pyramided plants are not deployed simultaneously. The single-gene plants would in that case provide a “stepping-stone” for pathogens to overcome each of the pyramided resistance genes (Zhao et al. 2003).

Cultivars with pyramided R_{Pi} -genes can be developed by traditional and time consuming breeding or with genetic modification. Genetic modification is assumed to be a fast way to insert two or more R_{Pi} -genes into an existing variety. A huge benefit is that undesirable linkage drag from the wild potato genome, causing for example an elevated glycoalkaloid content, is prevented. Most of the R -genes against *P. infestans* originate from crossable wild potato species. Therefore, the GM product should not be considered as transgenic but as cisgenic (Jacobsen and Schouten 2007). As long as public acceptance for genetic modification is lacking in the EU, classical introgression breeding is required. One positive aspect of the use of diploid progenitor lines is the high frequency of gene transfer of these genes to the tetraploid potato via 4X–2X crosses. On average 80% of the heterozygous R_{Pi} -gene in diploids will result in tetraploid offspring with a simplex R_{Pi} -gene due to First

Division Restitution of the unreduced male-gametes (Hermsen 1984). Molecular markers can facilitate the selection of superior descendants by tracing the R_{Pi} -genes in the breeding process and removing linkage drag efficiently.

In this paper, we have tested the hypothesis whether or not pyramiding is a valid strategy. There was no *a priori* expectation on the added value of the weaker $R_{Pi-mcd1}$ locus in the presence of the stronger R_{Pi-ber} locus. Our results suggest that pyramiding can result in an additive effect of the individual genes on the level of resistance. In our opinion, potato breeding can benefit from combining individual R_{Pi} -genes.

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