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Broad spectrum late blight resistance in potato differential set plants Ma*R8* and Ma*R9* is conferred by multiple stacked *R* genes

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Abstract *Phytophthora infestans* is the causal agent of late blight in potato. The Mexican species *Solanum demissum* is well known as a good resistance source. Among the 11 *R* gene differentials, which were introgressed from *S. demissum*, especially *R8* and *R9* differentials showed broad spectrum resistance both under laboratory and under field conditions. In order to gather more informa-
tion about the resistance of the *R8* and *R9* differentials, F1 and BC1 populations were made by crossing Mastenbroek (Ma) *R8* and *R9* clones to susceptible plants. Parents and offspring plants were examined for their pathogen recognition specificities using agroinfiltration with known *Avr* genes, detached leaf assays (DLA) with selected isolates, and gene-specific markers. An important observation was the discrepancy between DLA and field trial results for *Pi* isolate IPO-C in all F1 and BC1 populations, so therefore also field trial results were included in our characterization. It was shown that in Ma*R8* and Ma*R9*, respectively, at least four (*R3a*, *R3b*, *R4,* and *R8*) and seven (*R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, *R9*) *R* genes were present. Analysis of Ma*R8* and Ma*R9* offspring plants, that contained different combinations of multiple resistance genes, showed that *R* gene stacking contributed to the *Pi* recognition spectrum. Also, using a *Pi* virulence monitoring system in the field, it was shown that stacking of multiple *R* genes strongly delayed the onset of late blight symptoms. The contribution of *R8* to this delay was remarkable since a plant that contained only the *R8* resistance gene still conferred a delay similar to plants with multiple resistance genes, like, e.g., cv Sarpo Mira. Using this "de-stacking" approach, many *R* gene combinations can be made and tested in order to select broad spectrum *R* gene stacks that potentially provide enhanced durability for future application in new late blight resistant varieties.

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Introduction

Potato is the world's most important non-cereal food crop (Park et al. 2009; Rauscher et al. 2006). In many regions where potatoes are grown, one of the most notorious and economically damaging pathogens is *Phytophthora*

infestans (*Pi*), causing late blight (Halterman et al. 2008; Haverkort et al. 2008). The negative economic impact is $3.5 billion annually in developing countries alone (White and Shaw 2010). Fungicides and healthy seed tubers have been used to control potato late blight in developed countries. However, the use of expensive chemical sprays that have unfavorable environmental consequences, and the emergence of new fungicide resistant isolates, have increased the interest and use of host resistance in potato breeding programs (Grunwald et al. 2001; Fry and Goodwin 1997).

So far, over 20 functional late blight *R* genes, including the *Solanum demissum* genes *R1*, *R2, R3a*, and *R3b*, have been cloned (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; Li et al. 2011). The cloned genes all belong to the CC-NB-LRR class and, besides *S. demissum,* they were derived from wild *Solanum* species like *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005), *S. stoloniferum* and *S. papita*, (Vleeshouwers et al. 2008), *S. venturii* and *S. mochiquense* (Pel et al. 2009; Foster et al. 2009).

Solanum demissum, a hexaploid Mexican wild *Solanum* species, is an important source of resistance to late blight. The major resistance (*R*) genes from *S. demissum* show late blight race-specificity, which is associated with a hypersensitive response that is triggered by the cognate avirulence (*Avr*) genes of incompatible isolates of *Pi* (Vleeshouwers et al. 2011). Eleven *R* gene differentials (Black et al. 1953; Malcolmson and Black 1966; Eide et al. 1959) containing *R* genes introgressed into potato from *S. demissum,* have been collected by Mastenbroek (1952) and are referred to as the Mastenbroek differential set: Ma*R1* until Ma*R11*. Seven genes responsible for resistance within this set have been mapped: *R1* on chromosome 5 (Leonards-Schippers et al. 1992), *R2* on chromosome 4 (Li et al. 1998), *R3a, R3b*, *R6*, and *R7* on chromosome 11 (Huang et al. 2005; El-Kharbotly et al. 1996), and *R8* on chromosome IX (Jo et al. 2011). The differential set was originally thought to represent single late blight resistance factors (classified as *R1–R11*). However, *R1* was also found in the Ma*R5*, Ma*R6*, and Ma*R9* differentials (Trognitz and Trognitz 2007). Also the Ma*R3* differential plant contained two *R* genes, *R3a* and *R3b* (Huang et al. 2005).

In the past, *R* genes of Ma*R1*, *-2*, *-3*, *-4*, and *-10* from *S. demissum* have been introgressed into potato cultivars but were rapidly overcome by the *Pi* populations. In order to enhance durability of domesticated late blight resistance, stacking of two *R* genes, was shown to result in a delay in the onset of late blight disease (Tan et al. 2010). *R* gene stacking using conventional- or marker-assisted breeding is a complicated and time-consuming process due to the multitude of genes to be introgressed and due to the simultaneous introgression of undesired traits. Sarpo Mira is one of the few potato cultivars obtained by conventional breeding,

which expresses signicant levels of durable late blight resistance. Recently, it was shown that at least five *R* genes are stacked in cv Sarpo Mira (Rietman 2011). Besides traditional and marker-assisted breeding for late blight resistance, transformation of cloned *R* genes into existing potato varieties has gained renewed interest (Haverkort et al. 2008). Recently, it was shown that simultaneous transformation of multiple *R* genes into susceptible cultivars can be used to produce late blight resistant varieties (Zhu et al. 2011; Forch et al. 2010; BASF on line publication 2011).

Remarkably, among the set of 11 potato *R* gene differentials, Ma*R8* and Ma*R9* have shown a broad spectrum resistance in field trials as well as in detached leaf assays (DLA) against several complex isolates of *Pi* (Lehtinen et al. 2008). Lehtinen et al. proposed that this is probably due to a lack of selection pressure because *R9* has never been introduced into commercial cultivars. Zhang and Kim (2007) collected 261 *Pi* isolates during 3 years from Korea and found all races carried multiple virulence genes and showed virulence to the potato differential plants *R1*, *R3*, *R5*, *R6*, *R7*, *R10,* and *R11*, but not on *R8* and *R9*. Also Bisognin et al. (2002) found *Pi* isolates that were virulent on all *R* gene differentials except *R9* in DLA and on both *R8* and *R9* in field trials. More recently, it was shown that virulence towards *R8* and *R9* differential plants is relatively rare in European *Pi* populations (Chmielarz et al. 2010). In this and other studies (Corbiere et al. 2010; White and Shaw 2010) also cv Sarpo Mira, was included and was shown to express similar levels of resistance to late blight as the Ma*R8* and Ma*R9* in multiple years of field trials in Europe and Northern Africa.

Therefore, Ma*R8* and Ma*R9* differentials are expected to harbor important sources of late blight resistance and it is essential to know more about the genetic background of the resistance in these plants. A combination of DLAs with specific isolates, responsiveness to *Avr* genes and *R* gene-specific molecular markers was used to characterize resistance in Ma*R8* and Ma*R9* plants. We found that both Ma*R8* and Ma*R9* contained multiple resistance genes. In order to test whether broad spectrum resistance is a result of *R* gene stacking or a result of any of the individual *R* genes, plants with single *R* genes and plants with combinations of multiple *R* genes have been selected. Using an "on site" *Pi* virulence monitoring system, these plants were tested for their ability to resist high *Pi* infection pressure.

Materials and methods

Plant material

The Mastenbroek differential set Ma*R1, 2, 3, 4, 8,* and *9* (Mastenbroek 1952), cultivars Sarpo Mira, Concurrent,

Table 1 PCR primers used in this study for *R1*-, *R2*-, *R3a, R3b,* and *R8* diagnostic markers, and for *R2* and *R3a* homolog mining

Name	Sequence	Tm (°C)	Target gene	Product length (kb)	References
76-2SF2	CACTCGTGACATATCCTCACTA	50	*R1*	1.4 (Fig. 1)	Ballvora et al. (2002)
76-2SR	CAACCCTGGCATGCCACG				
R2-F1	GCTCCTGATACGATCCATG	50	*Rpi-abpt*	0.686 (Fig. 1)	This study
R2-R3	ACGGCTTCTTGAATGAA				
SHa-F	ATCGTTGTCATGCTATGAGATTGTT	56	*R3a*	0.982 (Fig. 1)	Huang et al. (2005)
SHa-R	CTTCAAGGTAGTGGGCAGTATGCTT				
CDP3	RRAGATTCAGCCATKGARATTAAGAAA		*R8*	0.500	Jo et al. (2011)
Adaptor pr.	ACTCGATTCTCAACCCGAAAG				
*R3b*F4	GTCGATGAATGCTATGTTTCTCGAGA	55	*R3b*	0.378 (Fig. 1)	Rietman (2011)
*R3b*R5	ACCAGTTTCTTGCAATTCCAGATTG				
R3-Clade1-F	caccATGGAGATTGGSTTARCARTTGGTGGTG	55	*R3*	2.5-4 (Fig. S2)	This study
R3-Clade1-R	AMAKRYATTYCCHATYGATNWBTATGGTGGRA		family		
R3-Clade2-F	caccATGGAKATTGGCTTWGCWGTTGGTGGKGC	55		3.5 (Fig. S2)	This study
R3-Clade2,3-R	AYWGGDATYYCMHATYRMTAWHTATGGTGGRRAT				
R3-Clade3,4-F	caccATGGADATTGGCTTAGCAGTTGGTKGT	55		2.5-4 (Fig. S2)	This study
R3-Clade4-R	TCACAGSYATTYCYCATYGATCTKTATAGTGGAA				
R2F	ATGGCTGATGCCTTTCTATCATTTGC	55	*R2*	2.5 (Fig. S2)	This study
R2R	TCACAACATATAATTCCGCTTC		family		

F forward, *R* reverse relative to ORF orientation

Bintje, and diploid clone RH89-039-16 (RH) were used for crosses and inoculation experiments. Concurrent is a descendent from the *R10* containing cultivar Estima (Vleeshouwers et al. 2000). F$_1$ individuals (two populations; *n* = 100) derived from the crosses Ma*R8* × Concurrent and Ma*R9* × Concurrent were produced and maintained in vitro at Wageningen UR plant breeding. 2-week-old in vitro propagated plants of both populations were transferred into the greenhouse. 2 to 4 weeks after transfer, DLA or agroin-filtration was performed.

R gene-specific markers

R1- and *R3a*-specific genetic markers were used for valida-tion of the presence or absence of these genes in Ma*R8* and Ma*R9* (Ballvora et al. 2002; Huang et al. 2005). The *R8*-specific marker was described by Jo et al. (2011) and is a cluster directed profiling marker (CDP3). *Rpi-abpt1* (an *R2* ortholog) specific molecular markers were developed based on alignments of *R2*, *Rpi-abpt*, *R2-like* (Lokossou et al. 2009) and nonfunctional homologs (Lokossou 2010). Stretches of inserted/deleted nucleotides were found, and three conserved primer sets were designed to detect *R2*. All three *R2* primer sets worked well with genomic DNA of Ma*R1*, Ma*R2*, Ma*R3*, Ma*R8*, Ma*R9*, cv. Concurrent, and RH89-039-16 (RH). The *R2* primer set that produced the longest polymerase chain reaction (PCR) product was selected (Table 1) and used in this study. Similarly, the *R3b* marker was designed after alignment of the recently cloned
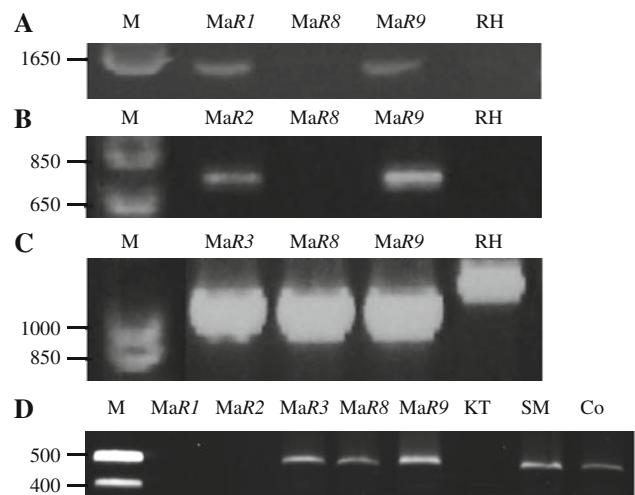

Fig. 1 *R* gene diagnostic markers. PCR products representing **a** *R1*-, **b** *R2*-, **c** *R3a*-, and **d** *R3b*-specific markers in Ma*R1*, Ma*R2*, Ma*R3*, Ma*R8,* and Ma*R9*, RH89-039-16 (RH), Kathadin (KT), Sarpo Mira (SM), and Concurrent (Co). On the left, the size of the DNA marker (M) in bp is provided. The 1,500 bp band produced by the *R3a* marker in RH was an a-specific product

R gene *R3b* (Li et al. 2011) with non-functional homologs. DNA was extracted from plantlets using a high-throughput method (Huang et al. 2005). The PCR primers listed in Table 1 were used in standard PCR reactions with the indi-cated annealing temperatures. PCR products were separated in a 1% agarose gel and stained with ethidium bromide (Fig. 1).

Phytophthora infestans assays

The DLA was performed according to Vleeshouwers et al. (2000) using the *Pi* isolates IPO-C, USA618, 99177, 90128, and 99189. These selected isolates were used because of their race specific virulence spectra. The maintenance and preparation of inoculum was according to previously published protocols (Vleeshouwers et al. 2000). Six days after inoculation, resistance versus susceptibility patterns were scored.

"On site" virulence monitoring was performed at three geographically isolated field locations in The Netherlands (Valthermond in the NorthEast, Lelystad in the center and Vredepeel in the SouthEast) in the years 2006–2010. All three locations represent important potato growing areas with high *Pi* infection pressure. Ma*R9*, Ma*R8* and the F1 plants Ma*R8*-18, Ma*R9*-43, Ma*R9*-89, and Ma*R9*-53 were analyzed. Bintje (*R0*), Sarpo Mira and Ma*R1* till Ma*R4*, were used as controls. All plants were propagated from in vitro culture and six plants per genotype were planted at each of the three different locations. This way, the plant material was exposed to the local *Pi* population and weather conditions from beginning of June to mid September without protection by fungicides. Every week, the plants were examined for potato late blight like symptoms. Infected plant material was sampled and used to obtain *Pi* pure cultures. To avoid a local epidemic, dominated by one or a few *Pi* genotypes, all six plants of a genotype were destroyed at the affected location as soon as two pure *Pi* cultures were recovered from this genotype.

The first blight observation date within an experiment was set as day 0. For each genotype, within each experiment and year the first late blight infection was observed and the number of days since the first observation in the field recorded. To estimate the delay in the onset of *Pi* symptoms for the different genotypes, the Censor method was used (Taylor 1973). For statistical analysis, the number of days delay since the start of the epidemic was $LOG10(x + 1)$ transformed. An observation is said to be censored if it is known only that it is less than (or greater than) a particular value. In this case, it is known that some genotypes were not infected during the sampling period. Therefore, the genotype remained free from infection by *Pi* for a fixed number of days (bound value) at a given location in a specific year. Therefore, it is known that the delay at this location and year is longer than the bound value, so this observation is censored. The censored observations were replaced by estimated values for delay, using the method outlined by Taylor (1973).

This method estimates the expected value of each censored observation iteratively conditional on the fact that the value must be greater than the fixed bound, and using the relevant information from the other observations in the experiment. Successively, a *T* test was performed to estimate significant differences between estimated delays of the different genotypes.

R2 and *R3a* allele mining

R2 allele mining primers were described by Lokossou et al. (2009; Table 1). The high fidelity proofreading DNA polymerase Phusion was used to amplify *R2*-like genes in the following PCR program: 98°C for 30 s. followed by 35 cycles of 98°C for 10 s, 62°C for 10 s and 72°C for 150 s, followed by 72°C for 10 min.

Based on a multiple sequence alignment containing *R3a* homologs from publically accessible databases, a phylogenetic tree was drawn and four clades could be distinguished (Figure S1). Clade-specific degenerate primers were designed and the sequence CACC was added to the 5′ end of the Forward primers to allow directional cloning into D-TOPO vector (Invitrogen, San Diego, CA, USA; Table 1). PCR reactions were done with Phusion™ DNA Polymerase using Ma*R9* and Ma*R8* genomic DNA as template (Figure S2). PCR conditions for *R3* clade one, two, and four specific primers: 30 s at 98°C, followed by 35 cycles of 98°C for 10 s, 65°C for 45 s, 72°C for 180 s, then followed by 72°C for 10 min. Using *R3* clade three specific primer: 30 s at 98°C, followed by 35 cycles of 98°C for 10 s, 62°C for 45 s, 72°C for 180 s; plus a final extension step at 72°C for 10 min.

Both the *R2* and *R3a* PCR products were purified by precipitation using 30% PEG8000/30 mM MgCl2 solution and was inserted into D-TOPO vector (Invitrogen). The recombinant plasmids were transformed into competent cells of *Escherichia coli* strain TOP10 and cultured in Luria-Bertani (LB) broth supplemented with Kanamycin (50 mg/ml). DNA sequencing was conducted on an ABI Prism 3700 DNA sequencer with primer M13F and *R2*- and *R3a*-derived primers inside of inserts.

Sequence similarity was analyzed with Seqman v.7 software (Lasergene, DNAStar, Madison, WI, USA). Sequence comparison was performed with MEGALIGN v.7.

Agroinfiltration assay

Fifteen days after transfer into the greenhouse, F1 population plants derived from Ma*R8* and Ma*R9* and their controls were used for agroinfiltration experiments with *Avr1*, *Avr2*, *Avr3a,* and *Avr4*. Safety regulations for containment determined the use of a separate greenhouse compartment for agro-treatments. 3, 5, and 7 days after agroinfiltration, symptoms which are caused by the interaction between *R* genes and the corresponding *Avr* genes were observed and scored. Agroinfiltration assay followed previously described methodology (Vleeshouwers et al. 2008).

Table 2 On site *Pi* virulence monitoring

Year	Genotype	*R* gene content	Calculated delay in *Pi* symptoms (days)	Relative delay in *Pi* symtoms*
2006	Bintje	–	4	`a . .`
	Ma*R1*	1	4	`a . .`
	Ma*R2*	2	8	`a b .`
	Ma*R3*	3a, 3b	4	`a . .`
	Ma*R4*	4	7	`a b .`
	Ma*R8*	3a, 3b, 4, 8	37	`. b .`
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	66	`. . c`
2007	Bintje	–	1	`a . . .`
	Ma*R1*	1	2	`a b . .`
	Ma*R2*	2	2	`a b . .`
	Ma*R3*	3a, 3b	2	`a b . .`
	Ma*R4*	4	1	`a . . .`
	Ma*R8*	3a, 3b, 4, 8	13	`. . c d`
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	`. . . d`
2008	Bintje	–	3	`a b . .`
	Ma*R1*	1	1	`a . . .`
	Ma*R2*	2	3	`a b . .`
	Ma*R3*	3a, 3b	2	`a b . .`
	Ma*R4*	4	2	`a b . .`
	Ma*R8*	3a, 3b, 4, 8	24	`. . c d`
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	15	`. . c .`
2009	Bintje	–	1	`a . .`
	Ma*R8*	3a, 3b, 4, 8	113	`. b .`
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	`. . c`
	Sarpo mira	3a, 3b, 4, smira1, 2	No lesions**	`. . c`
2010	Bintje	–	3	`a . . .`
	R8-18	8	50	`. b . .`
	Ma*R8*	3a, 3b, 4, 8	50	`. b . .`
	Sarpo mira	3a, 3b, 4, smira1, 2	51	`. b . .`
	R9-53	3a, 3b, 4, 8, 9	193	`. . c .`
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	`. . . d`

* The onset of *Pi* symptoms was compared between different genotypes using *T* test. Significant differences in delay between different genotypes are represented by different letters. If ranges of letters are overlapping there was no significant difference observed between the genotypes in this particular year

** In case no lesions were found on the tested plants during the course of the experiment, calculations for delay according to censor showed an unrealistic figure, which was replaced by "no lesion"

Results

Low virulence levels in *Pi* populations in the Netherlands towards Ma*R8* and Ma*R9* plants

In order to study whether Ma*R8* and Ma*R9* plants expressed useful resistance to the *Pi* populations in The Netherlands, an "on site virulence monitoring" system was set up. Cv Bintje (R0) was included as reference plant that lacks *Pi* resistance and cv Sarpo Mira was included in the last 2 years of these studies as reference plant with a high level of *Pi* resistance. In addition, four plants from the Mastenbroek differential set were included (Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4*) because their *R* gene content is well described. In all 5 years both Ma*R8* and Ma*R9* performed significantly better than cv Bintje, Ma*R1,* Ma*R2,* Ma*R3,* and Ma*R4* plants, showing that useful resistance is present in Ma*R8* and Ma*R9* plants (Table 2). In 4 of the 5 years, Ma*R9* showed a significantly longer delay in the onset of *Pi* symptoms when compared with Ma*R8*. In 2009 and 2010 cv Sarpo Mira was also included and Ma*R9* and Ma*R8* showed a similar or even longer delay in the onset of *Pi* symptoms. Despite the observed variations per year, it can be concluded that Ma*R8* and Ma*R9* express high levels of resistance to severe *Pi* challenges.

Multiple known *R* genes are stacked in Ma*R8* and Ma*R9* plants

In order to determine the recognition specificity of the resistance in Ma*R8* and Ma*R9*, the plants were screened for their ability to recognize any of the known Avr genes. Using an agroinfiltration assay *Avr1*, *Avr2*, *Avr3a*, and *Avr4* were infiltrated into Ma*R8* and Ma*R9* plants (Fig. 2a;

Table 3 Avr responses and *R* gene-specific markers in selected Mastenbroek differential set plants, susceptible, and resistant cultivars

	Ma*R1*	Ma*R2*	Ma*R3*	Ma*R4*	Ma*R8*	Ma*R9*	Bintje (*R0*)	Concurrent
Avr1	HR	n	n	n	n	HR	n	n
Avr2	n	HR	n	n	n	HR	n	n
Avr3a	n	n	HR	n	HR	HR	n	n
Avr4	n	n	n	HR	HR	HR	n	n
R3a + Avr3a	HR	HR	HR	HR	HR	HR	HR	HR
pGraB	n	n	n	n	n	n	n	n
R1	+	−	−	−	−	+	−	−
R2	−	+	−	−	−	+	−	−
R3a	−	−	+	−	+	+	−	−
R3b	−	−	+	−	+	+	−	+
R8	−	−	−	−	+	+	−	−

HR hypersensitive response, *n* no hypersensitive response, + presence of specific marker band, − absence of specific marker band

Table 3). Interestingly, Ma*R8* showed an HR reaction with *Avr3a* and *Avr4*. Ma*R9* showed a HR upon infiltration with all tested *Avr* genes; *Avr1*, *Avr2*, *Avr3a*, and *Avr4* (Table 3). These data show that a broad spectrum of *Avr*'s is recognized, which could be explained by the presence of multiple *R* genes or by broad spectrum *R* genes that recognize multiple *Avr*'s.

In order to study whether multiple or single broad spectrum *R* genes were present in the studied plants, Ma*R8* and Ma*R9* were crossed with cv Concurrent. F1 populations were sown and maintained in vitro. Agroinfiltrations of *Avr1*, *Avr2*, *Avr3a*, and *Avr4* were performed in these F1 populations. Segregation patterns were different for each *Avr* (Fig. 2b, Table S1) and it is concluded that different *R* genes must be responsible for their recognition. Because *Avr1*, *2*, *3a*, and *4* are known to be recognized by *R1*, *R2*, *R3a*, and *R4*, our results suggest that *R3a* and *R4* genes are present in Ma*R8* and *R1*, *R2*, *R3a*, and *R4* are present in Ma*R9*. It is, however, an unexpected result since the Ma*R8* and Ma*R9* plants have been selected as *R* gene differentials and were assumed to contain only one *R* gene. In order to confirm the presence of multiple *R* genes in these plants, the molecular basis of the observed effector recognition was investigated.

Solanum demissum late blight *R* genes *R1*, *R2*, and *R3a* have been cloned and their sequences have been used to develop gene-specific molecular markers. The previously published *R1*- and *R3a*-specific markers (Trognitz and Trognitz 2007; Huang et al. 2005) and the *R2*-specific marker (this study) were used to monitor the presence of these genes in Ma*R8*, Ma*R9*, cv Concurrent and RH (Fig. 1), respectively. Also markers derived from the recently cloned *R3b* gene (Li et al. 2011) and the recently mapped *R8* gene (Jo et al. 2011) were included in this study. Ma*R8* was positive for the *R3a*, *R3b*, and *R8* markers. Ma*R9* showed to be positive for the *R1*, *R2*, *R3a*, *R3b*,
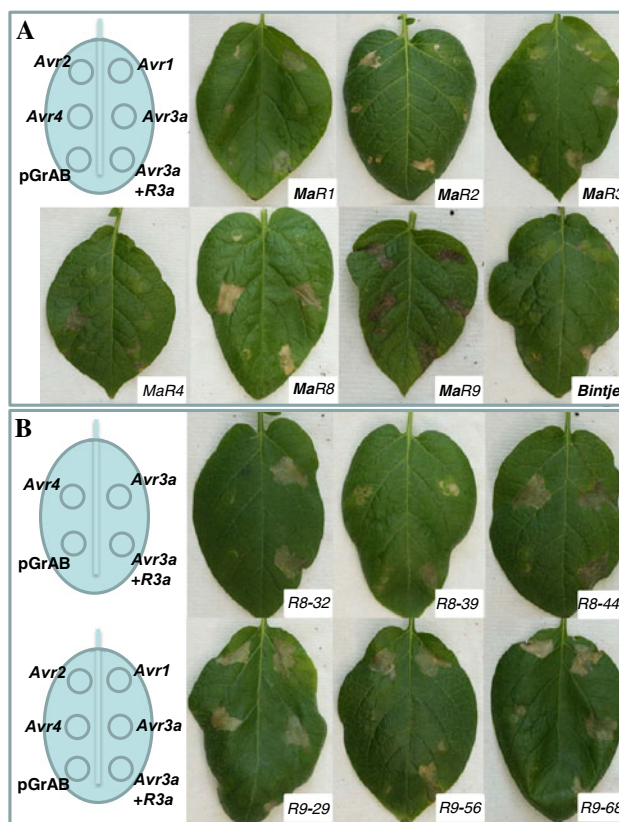

Fig. 2 *Avr1, -2, -3a,* and *-4* induced hypersensitive response. *Avr1, -2, -3a,* and *-4* were agroinfiltrated in Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4*, Ma*R8*, Ma*R9,* and Bintje (**a**) or Ma*R8* and Ma*R9* F1 offspring plants (**a**). pGraB and 1:1 mixture of *R3a* and Avr3a were infiltrated as negative and positive controls, respectively. Representative leaves from three biological replicates are shown

and *R8* markers. Remarkably, also cv Concurrent did show the *R3b* marker band. Cv Concurrent and RH were negative for the *R1*, *R2*, *R3a*, and *R8* markers. Therefore, Ma*R8* is expected to contain *R3a*, *R3b*, and *R8*. Ma*R9* is expected to contain *R1*, *R2*, *R3a*, *R3b*, and *R8* or closely related homologs

Table 4 Segregation of *R* gene diagnostic markers, Avr4 responses and disease resistance in F1 and BC1 populations

	F1		BC1	
	3020	3025	3150	3153
	Ma*R8*×C	Ma*R9*×C	*R9*-43×K	*R9*-53×K
R1	Absent	51:49:0		
Rpi-abpt1	Absent	62:38:0		
R3a	49:45:6	88:11:1**		
R3b	62:28:0	88:8:0		
R4 (Avr4)	59:34:7	56:20:24*		
R8	56:44:0	38:51:7**	4:6:0	8:9:0
IPO-C field	55:43:1	95:1:4	4:6:0	12:5:0

Segregation ratios given represent responsive:inresponsive:questionable for Avr4 responses, present:absent:questionable for marker scores, and resistant:susceptible:questionable for disease scores. Data are derived from Table S1

* High number of questionable scores are most likely caused by the strong response to agroinfiltration treatment in this population

** Questionable scores for the *R3a* and *R8* markers were caused by fragments migrating at the same position as the marker bands but had weaker intensities as observed in the other genotypes

thereof. This notion was confirmed upon analysis of the F1 populations. When segregation patterns of *Avr* responsiveness were compared to *R* gene marker segregation patterns a co-segregation was observed (Table S1). Plants that produced a high background cell death response to agroinfiltration and plants that responded poorly to agroinfiltration were excluded from the comparative analyses. *R* gene marker segregation ratios are shown in Table 4. For *R4* no marker was available and here segregation ratios were inferred from effector responsiveness only. It can be concluded that Ma*R8* contains *R3a*, *R3b*, *R4*, and *R8* in simplex. Overrepresentation of *R3b* in the *R8* population is caused by the presence of *R3b* in the male parent cv Concurrent. In Ma*R9; R1, R2, R4,* and *R8* genes seemed to be present in simplex. *R3a* and *R3b* seemed to be present in duplex.

In order to further confirm the presence of the known *R* genes in the plants under study, a *R2* and *R3* (covering both *R3a* and *R3b*) homolog mining approach was pursued. Using primer pairs overlapping the start and stop codons of *R2* and *R3*, dozens of open reading frame (ORF) sequences with high levels of homology to *R2* and *R3,* respectively, were identified in Ma*R8* and Ma*R9* plants. Using the *R2* primers, six clones were identified deriving from Ma*R9*, one clone (9-R-8) was identical at the nucleotide level to *Rpi-abpt1* (Fig. 3). Using the *R3* primers, among 24 clones two exact copies (9-G-52 and 9-G-81) of *R3a* were found in Ma*R9* and no clones identical to *R3b* were found in Ma*R9*. In Ma*R8*, among 6 clones, no exact copies of either *R3a* or
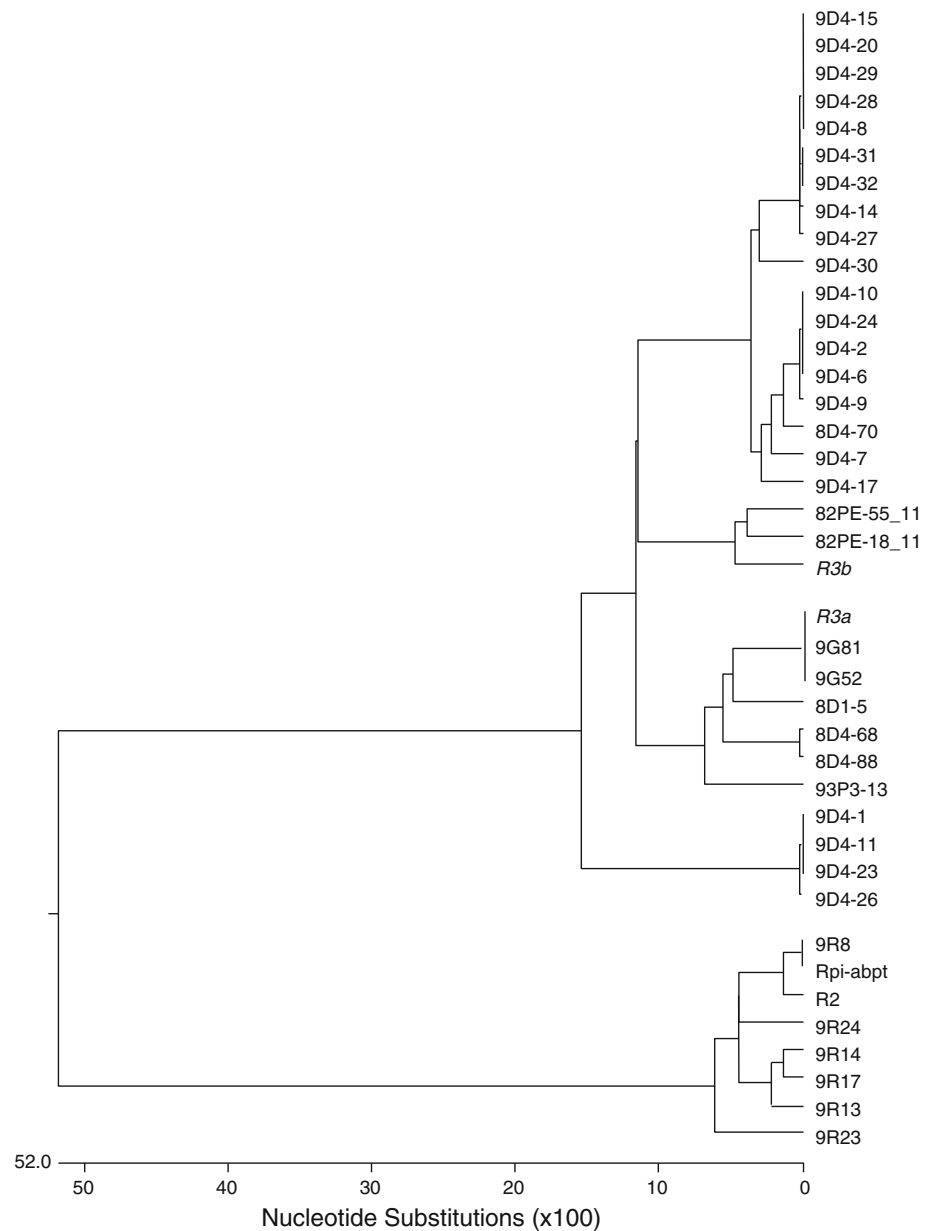
R3b were found (Fig. 3). The absence of *R3a* in the screening of Ma*R8* and the absence of *R3b* in the screening of both Ma*R8* and Ma*R9* is considered to be due to the low saturation of the screen and does not show that *R3a* or *R3b* are absent in those plants. These results suggest that the *Avr2* and *Avr3a* recognition by the Ma*R9* plants is caused by *Rpi-abpt1* and *R3a,* respectively. Also, because of the sequence identity, it can be ruled out that wider *Avr* spectra are recognized by these individual genes to cause the broad spectrum resistance of the Ma*R9* plants.

R8 and at least one additional *R* gene is present in Ma*R9*

Since it was found that multiple known *R* genes are present in Ma*R8* and Ma*R9*, the possibility of additional, unknown, late blight *R* genes was also investigated. Because of the presence of the multitude of known *R* genes in the studied plants, it was decided to study the populations using a *Pi* isolate, IPO-C, that had overcome *R1*, *R2*, *R3a*, *R3b*, and *R4*, leaving the possibility to study additional resistance genes in Ma*R8* and Ma*R9*. Although the parental plants were resistant to IPO-C in our DLAs, analysis of the F1 populations revealed no (in the case of Ma*R8*) or only very few plants (in the case of the Ma*R9* F1 population) that consistently showed resistance to IPO-C in DLA. However, when the Ma*R8* and Ma*R9* populations were tested in field trials in 2009 and 2010, inoculated with the same isolate (IPO-C), 2 to 4 weeks after inoculation a clear distinction could be made between resistant and susceptible plants. Only a few plants could not be classified as resistant or susceptible and were qualified as questionable (Table 4, Table S1).

It had been described that resistance to IPO-C in the Ma*R8* F1 population, as determined under field conditions, showed a 1:1 segregation and fully co-segregated with the *R8* marker (Jo et al. 2011). The segregation of IPO-C resistance in the Ma*R9* F1 population was severely skewed towards resistance, suggesting that multiple unlinked *R* genes were present. Interestingly, the *R8* marker segregated in a 1:1 fashion (Table 4) in the Ma*R9* F1 population and all the positive plants were resistant to IPO-C. In addition out of 51 plants that lacked the *R8* marker, 46 plants were fully resistant to IPO-C, showing that at least one additional *R* gene is segregating in the population. In order to reduce the *R* gene complexity, BC1 populations were generated deriving from Ma*R9*. Eight resistant F1 plants were selected and crossed with susceptible cultivar Kathadin. The BC1 populations were tested in a field trial inoculated with IPO-C. Two BC1 populations showed a 1:1 segregation for resistance and four populations showed a skewing towards resistance. So, even after crossing to a susceptible plant twice, still a multitude of resistance was observed, deriving from Ma*R9*. The *R8* marker and IPO-C

Fig. 3 Phylogenetic analysis of the *R3a* and *R2* homologs. Full-length sequences from allele mining studies with 80% or more nucleotide identity to *R3a* and *R2* were aligned with known *R3a* and *R2* homologs using Megalign from the DNAstar software pack. Sequence names starting with 8 or 9 are derived from Ma*R8* or Ma*R9,* respectively


```
                                                           9D4-15
                                                           9D4-20
                                                           9D4-29
                                                           9D4-28
                                                           9D4-8
                                                           9D4-31
                                                           9D4-32
                                                           9D4-14
                                                           9D4-27
                                                           9D4-30
                                                           9D4-10
                                                           9D4-24
                                                           9D4-2
                                                           9D4-6
                                                           9D4-9
                                                           8D4-70
                                                           9D4-7
                                                           9D4-17
                                                           82PE-55_11
                                                           82PE-18_11
                                                           R3b
                                                           R3a
                                                           9G81
                                                           9G52
                                                           8D1-5
                                                           8D4-68
                                                           8D4-88
                                                           93P3-13
                                                           9D4-1
                                                           9D4-11
                                                           9D4-23
                                                           9D4-26
                                                           9R8
                                                           Rpi-abpt
                                                           R2
                                                           9R24
                                                           9R14
                                                           9R17
                                                           9R13
                                                           9R23
  52.0
        50      40      30      20      10       0
            Nucleotide Substitutions (x100)
```

resistance segregation ratios in two selected BC1 populations (3150 and 3153) are shown in Table 4. A co-segregation from the *R8* marker and IPO-C resistance was observed in population 3150. This showed that the *R8* marker not only tagged a *R* gene from Ma*R8* but also from Ma*R9* and this gene is referred to as *R8* since it is most likely the same gene as the *R8* gene derived from Ma*R8*. In population 3153, that was skewed towards resistance, the *R8* marker was present exclusively in resistant plants, showing that the *R8* gene was also segregating in this population. Also a second *R* gene, deriving from Ma*R9*, was segregating in population 3153 since four plants without the *R8* marker also showed resistance (Table 4). The gene(s) responsible for this resistance is tentatively referred to as *R9*.

Contribution of *R* gene stacking to broad spectrum resistance

The *R* genes within Ma*R8* and Ma*R9* were genetically segregating in different F1 and BC1 populations. This allowed the selection of plants with different combinations of *R* genes that can be used to test for the contribution of each of these *R* genes to *Pi* isolate recognition spectrum. In Table 5, the *R* gene content of selected F1 plants is summarized as derived from *R* gene-specific markers, effector recognition, and IPO-C resistance segregation in field trials.

Five different *Pi* isolates were tested on these plants. As described above and in previous studies, F1 plants containing *R8* and *R9* genes showed no or only weak resistance to IPO-C in DLA. Plant Ma*R9*-71 was an exception. Like the

Table 5 Resistance spectra of selected differential set plants and of Ma*R8*, Ma*R9* F1 plants

| | R gene | 90128 | 99189 | USA618 | 99177 | IPO-C | IPO-C |
	Content	(Avr2)	(Avr3a, Avr4)	(Avr4)		DLA	Field
Ma*R1*	1	S	S	S	S	S	S
Ma*R2*	2	R	S	S	S	S	S
Ma*R3*	3a, 3b	S	R	S	S	S	S
Ma*R4*	4	S	R	R	S	S	S
Ma*R8*	3a, 3b, 4, 8	S	R	R	R	R	R
Ma*R9*	1, 2, 3a, 3b, 4, 8, 9	R	R	R	R	R	R
Concurrent	3b	S	S	S	R	S	S
Bintje	0	S	S	S	S	S	S
F1							
R8-18	8	S	S	S	nd	S	R
R8-69	4, 8	S	R	R	nd	S	R
R8-93	3a, 3b, 8	S	R	S	nd	S	R
R9-12	3b, 4, 9	S	R	R	R	S	R
R9-21	2, 3a, 3b, 9	R	R	S	R	S	R
R9-43	3a, 3b, 8	S	R	S	R	q	R
R9-53	2, 3a, 3b, 8, 9	R	R	S	R	q	R
R9-56	1, 2, 9	R	S	S	R	S	R
R9-71	2, 3b, 4, 9	R	R	R	R	R	R
R9-72	2, 3a, 3b, 4, 9	R	R	R	R	S	R
R9-89	3a, 8, 9?	S	R	S	R	S	R

A representative selection of the F1 populations is shown. Resistance to IPO-C was also tested under field conditions. Raw data are presented in Table S1

R resistant, *q* weak resistance or inconsistent results, *S* susceptible, *nd* no data available, *9?* it could not be determined if also *R9* was present in this plant, besides *R8*

parental plant Ma*R9*, this plant did express resistance to IPO-C in DLAs (Table 5). Due to this lack of sufficient biological activity of the *R8* and *R9* genes in DLA, the biological activity of the remaining genes *R2*, *R3a*, *R3b*, and *R4* could be distinguished by the use of differential isolates. None of the selected plants, except *R9*-56, contained *R1* so no discriminating isolate was used for *R1*. Isolate 90128 was avirulent on the plants containing *Rpi-abpt1*. This is in agreement with previous studies and confirms that 90128 expresses *Avr2*. Isolate USA618 was avirulent on all plants containing *R4*, suggesting that this isolate expresses *Avr4*. Isolate 99189 was avirulent on all plants containing *R3a* and *R4*. By comparison to the resistance spectrum of USA618, 99189 could be used as a differential isolate for *R3a*. Isolate 99177 was avirulent on cv Concurrent that was used as the "susceptible" parent for the Ma*R8* and Ma*R9* populations. Resistance to isolate 99177 largely co-segregated with the marker for *R3b* that was derived from parent cv Concurrent. It could, however, not be concluded that isolate 99177 expressed *Avr3b* since it was compatible on Ma*R3* plants that also contained the *R3b* gene. Based on these studies, it can be concluded that stacking of *R2*, *R3a*, *R3b*, and *R4* genes contributes to the isolate resistance spectrum of the plants. It remains, however, to be resolved to what extent stacking of these genes contributes to durability of resistance.

Contribution of *R* gene stacking to durability; a new method to study durability of *R* gene stacks

As described above, the Ma*R8* and Ma*R9* plants showed resistance to high *Pi* infection pressure in "on site" virulence monitoring fields from 2006 to 2010 in three different potato growing areas in the Netherlands. This resistance was similar to the resistance in the durably resistant cultivar Sarpo Mira. In order to set up an *R* gene stack durability assay, the F1 plants from the Ma*R8* and Ma*R9* populations, that contained reduced levels of *R* gene stacking, can be monitored in the same "on site" virulence monitoring fields as the parental plants Ma*R8* and Ma*R9*. In 2010, a first trial was performed (Table 2) and Ma*R8* showed an indistinguishable delay in the onset of late blight symptoms as plant *R8*-18 which contained, besides *R8,* no additional known *R* genes. This suggests that there was no significant

contribution from the additional *R* genes in Ma*R8*. It must be noted that this is only the first year of these trials. Similar experiments in the years to come must be performed in order to assess whether these findings will show consistency. Remarkable is the finding that cv Sarpo Mira, which contains at least five *R* genes (*R3a*, *R3b*, *R4*, *Rpi-smira1,* and *Rpi-smira2*; Rietman 2011), had a similar delay in the onset of *Pi* symptoms as Ma*R8* and *R8*-18 in 2010. However, in 2009 cv Sarpo Mira remained unaffected while Ma*R8* did show *Pi* symptoms towards the end of the experiment. This showed that differences in virulence levels towards *R* genes in the *Pi* population can vary from year to year. The virtue of *R* gene stacking was illustrated by plant Ma*R9*-53, that besides *R8* also contained *R9*. Ma*R9*-53 showed a significantly prolonged delay in the onset of *Pi* symptoms. The delay in Ma*R9*-53 remained, however, below that of Ma*R9*, which remained unaffected in 2010. This suggests that besides *R8* and *R9* additional factors in Ma*R9* were enhancing the resistance to *Pi*.

Discussion

Molecular basis of resistance in Ma*R8* and Ma*R9*

Ma*R8* and Ma*R9* were exposed in sequential years to high *Pi* infection pressure in trap fields in major potato growing areas in the Netherlands. The onset of late blight symptoms was observed and in every year Ma*R8* and Ma*R9* performed significantly better then control cultivar Bintje and in most cases also significantly better then Ma*R1*, Ma*R2*, Ma*R3,* and Ma*R4* plants (Table 2). In the last 2 years, also comparisons were made to cv Sarpo Mira. In 2009, Ma*R9* showed a similar delay as cv Sarpo Mira, and in 2010 Ma*R8* showed a similar delay as cv Sarpo Mira. In 2010, Ma*R9* even outperformed cv Sarpo Mira. This was in agreement with other comparisons made between Ma*R8*, Ma*R9* and for cv Sarpo Mira in Western Europe and Northern Africa (Chmielarz et al. 2010; Corbiere et al. 2010; White and Shaw 2010). Using a combination of studies including *Avr* responsiveness, *R* gene-specific molecular markers, candidate gene cloning and segregation analysis of these traits in F1 populations, it was possible to show that *R3a*, *R3b*, *R4,* and *R8 S. demissum* late blight resistance genes were present in Ma*R8* and that *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8, R9* and potentially an additional *R* gene were present in Ma*R9*. This (these) *R9* gene(s) must not be confused with the resistance provided by Ma*R9* and therefore it is only tentatively referred to as *R9*. The additional resistance in Ma*R9* requires further research to identify its molecular and genetic basis. Complicating for this approach will be the difficulty to detect *R9* resistance using DLA in the current F1 and BC1 populations.

R8 and *R9* resistance is background dependent

In F1 plants, a remarkable discrepancy was found between resistance to IPO-C in DLA and in field trials. Parental plants Ma*R8* and Ma*R9* were resistant to IPO-C in DLA and field trials, whereas, only a few F1 plants were consistently resistant to IPO-C in DLA. In field trials, however, at least half of the F1 plants were found to be consistently resistant. Also for cv Sarpo Mira F1 populations, it was reported that DLA resistance to IPO-C was not inherited properly, whereas resistance to IPO-C in field trial conditions was inherited as a dominant trait (Rietman 2011). It has been described before that *R* genes can be poorly segregating traits (Ordonez et al. 1997; Trognitz 1998). Dominant-, resistance-suppressing factors that can be segregating in the same population have been postulated to explain this phenomenon (El-Kharbotly et al 1995). In our populations, such suppressor genes may have derived from the susceptible parent Concurrent. On the other hand, poor resistance segregation may have been caused by multiple accessory genes, deriving from the resistant parent. These accessory genes, required for resistance, may have been present in a heterozygous state and are, therefore, not all present in all offspring plants (Ordonez et al. 1998). Such accessory genes could encode guardees, host components that are guarded (Jones and Dangl 2006) by the R8 or R9 proteins. Alternatively, they could encode downstream signaling components or upstream transcription factors required for the proper transcriptional regulation of the *R8* and *R9* genes. This hypothesis implies that the *R8* and *R9* genes might not be sufficiently active in all genetic backgrounds. Indeed, populations have been found in which the *R8* gene is present and segregating but resistance is only expressed to a very limited extent in field trial conditions (JV, unpublished data). The postulated background dependence of *R8* and *R9* has important implications for GM- and marker-assisted breeding strategies in which multiple broad spectrum *R* genes are stacked. It can be concluded that additional tools need to be developed to determine and distinguish the biological activity of *R* genes in stacks.

Extensive *R* gene stacking in Ma*R8* and Ma*R9* caused broad spectrum resistance

It was shown recently that *R2* and *R3a* homologs could have divergent Avr recognition and, therefore, altered *Pi* recognition spectra (Champouret 2010). This gives rise to the question whether the *R2* and *R3* homologs in the studied plants are responsible for the observed broad spectrum resistance. Cloning of the *R* genes responsible for the recognition of Avr2 and Avr3a from Ma*R9* plants showed sequence identity to *Rpi-abpt1* and *R3a,* respectively. Sequence identity between the genes will result in identical

proteins and identical recognition specificities, ruling out the possibility that either of these two genes caused the broad spectrum resistance observed in Ma*R9*.

A better explanation for the broad spectrum resistance was found in the *R* gene stacks observed in Ma*R8* and Ma*R9* plants. F1 and BC1 studies revealed the presence of plants with many different combinations of the *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8,* and *R9* genes. These different combinations resulted in distinct isolate resistance spectra (Table 5). An interesting observation was the resistance spectrum of cv Concurrent, which was used as crossing parent with Ma*R8* and Ma*R9*. This cultivar carries the resistance of Ma*R10* because of its parent cv Estima (Vleeshouwers et al. 2000), which can account for its resistance to isolate 99177. In cv Concurrent, we also found the presence of *R3b*, which locates, like *R10,* to the distal end of the short arm of chromosome 11. It was shown recently that in Ma*R10*, *R3b* and *R10* are two different, closely linked genes (Rietman 2011). Therefore, it cannot be ruled out that plants carrying the *R3b* marker, are in fact containing the *R3b* and *R10* genes, contributing to a different isolate resistance spectrum as plants carrying *R3b* and not *R10*.

Durability in relation to stacking

It is shown that the more *R* genes are present in a plant the broader the isolate resistance spectrum is (Table 5). Theoretically, broad spectrum or multi-*R* gene-based resistance, would result in enhanced durability because it is increasingly less likely that all cognate *Avrs* are lost simultaneously in one *Pi* spore. In the *Pi* population in the Netherlands, however, virulence towards plants containing *R1*, *Rpi-abpt1*, *R3a*, *R3b,* and *R4* is very common (Table 2). When F1 clone *R8*-18, that only contained *R8* and not *R3a*, *R3b,* and *R4*, was analyzed by "on site" virulence monitoring, no difference was observed with the Ma*R8*. Although this is only a 1 year observation, the fact that this was observed at three different locations suggests that the additional *R* genes provided no or minor contribution to the resistance spectrum towards the *Pi* population in the Netherlands. Recent data show that also in cv Sarpo Mira, which has remained durably resistant already for several years, at least five *R* genes are stacked (*R3a*, *R3b*, *R4*, *Rpi-smira1,* and *Rpi-smira2*; Rietman 2011).

The observation that *R8* has a major contribution to resistance in Ma*R8* and Ma*R9* can be explained in two ways. A potential *Avr8* is hard to lose for *Pi* or, the worldwide *Pi* population has not been "trained" yet to evade *R8* recognition because *R8* has not been applied extensively yet in potato varieties. Also a combination of both arguments could hold. In order to avoid *Pi* populations to mutate and evade *R8* recognition, it would be wise to stack additional *R* genes that still show broad spectrum resistance

on top of the *R8* gene. The Ma*R9* F1 population described in this study can be used to select for additional *R* genes that combine well with *R8*. Individuals with different known and unknown *R* gene combinations will be selected and will be tested by "on site" virulence monitoring. This will prove an important way to select for *R* gene stacks with potentially improved durability. These stacks could, successively, be selected in marker-assisted breeding programs or could be reconstituted in a GM approach (Jacobsen and Schouten 2007).

Besides cv Sarpo Mira, recently two other potato cultivars have been described to contain durable late blight resistance. C88 is a cultivar that is grown in Asian countries already for more than 10 years and it is now the number one variety in the Chinese province Yunnan due to its late blight resistance (Li et al. 2010). Also the US cultivar Missaukee shows durability already for 7 years (Douches et al. 2010). Of course, durability must prove over the years to come, but also for these two cultivars it would be appropriate to identify the molecular basis of resistance aiding the design of strategies for prolonged durability. Such a strategy could consist of monitoring of virulence within the local *Pi* populations. Virulence towards the *R* genes involved can be detected and can be linked to a fungicide spray advice.

Implications for the *R* gene differential set

A system of tester plants with one *R* gene is essential to determine the virulence spectrum of individual *Pi* isolates. The differential set Ma*R1* till Ma*R11* was long considered to contain one *R* gene per tester plant. However, research during cloning of the *R3* gene clearly indicated that the differential plants from Mastenbroek and Black were different. Ma*R3* contained two resistance genes, *R3a* and *R3b*, and the Bl*R3* differential contained only *R3a* (Huang et al. 2005). Trognitz and Trognitz (2007) showed that the differentials Ma*R5*, Ma*R6,* and Ma*R9* all contain the *R1* gene. The availability of cloned *R* genes and/or matching *Avr* genes enabled to test also the Ma*R8* and Ma*R9* differentials for *R* gene stacking. In this study five *R* gene-specific markers (*R1, R2, R3a, R3b,* and *R8*) and four *Avr* genes matching with *R1, R2, R3a,* and *R4* were used. Unexpectedly, three additional *R* genes were found in Ma*R8* and at least five additional *R* genes were found in Ma*R9*. According to our data, race 9 isolates are predicted to also be race 1, 2, 3, 4, 8. Race 8 isolates are predicted to be also race 3, 4. In literature, however, race combinations are found that seem erroneous in retrospect. Consequently, past and future race typing of *Pi* isolates might require careful (re)evaluation using the current knowledge.

Our unpublished data show that also Ma*R5*, Ma*R6*, and Ma*R7* contain multiple *R* genes. Moreover, Rietman (2011)

showed that Ma*R10* contains *R3b* and *R10*. These findings call for an update of the differential set. An improved or "purified" differential set will allow a more accurate analysis of *Pi* isolate virulence spectra. This could be done by crossing the original differential set plants or their offspring to cultivars without *R* genes. So far, it was possible to select a plant that contained only *R8* (*R8*-18) but a plant with only *R9* remains to be identified. Additional BC2 crosses will be required for this. Such "purified" differential plants will be very useful for future "on site" virulence monitoring. In parallel to an improved differential set, virulence towards *R* genes could be monitored using PCR assays targeting known *Avr* genes. If an *Avr* gene is lost in *Pi* isolates from a geographic location in a potato growing season, this is a good indication that virulence towards the matching *R* gene in the potato population has emerged. Rapid PCR (a)-virulence assays could be linked to fungicide spray advice in a defined geographic region in order to reduce fungicide application dosage and frequency.

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