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Qualitative and Quantitative Late Blight Resistance in the Potato Cultivar Sarpo Mira Is Determined by the Perception of Five Distinct RXLR Effectors

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Potato defends against *Phytophthora infestans* infection by resistance (*R*)-gene-based qualitative resistance as well as a quantitative field resistance. *R* genes are renowned to be rapidly overcome by this oomycete, and potato cultivars with a decent and durable resistance to current *P. infestans* populations are hardly available. However, potato cultivar Sarpo Mira has retained resistance in the field over several years. We dissected the resistance of 'Sarpo Mira' in a segregating population by matching the responses to *P. infestans* RXLR effectors with race-specific resistance to differential strains. The resistance is based on the combination of four pyramided qualitative *R* genes and a quantitative *R* gene that was associated with field resistance. The qualitative *R* genes include *R3a, R3b*, *R4*, and the newly identified *Rpi-Smira1*. The qualitative resistances matched responses to avirulence (AVR)3a, AVR3b, AVR4, and AVRSmira1 RXLR effectors and were overcome by particular *P. infestans* strains. The quantitative resistance was determined to be conferred by a novel gene, *Rpi-Smira2*. It was only detected under field conditions and was associated with responses to the RXLR effector AvrSmira2. We foresee that effector-based resistance breeding will facilitate selecting and combining qualitative and quantitative resistances that may lead to a more durable resistance to late blight.

Potato, the third largest global food crop after wheat and rice, suffers from the devastating late blight disease that results in global yield losses of 16% (Haverkort et al. 2009). Late blight is caused by the oomycete *Phytophthora infestans*, which is renowned for triggering the Irish potato famine in the 1840s. The most sustainable strategy to protect potato plants from late blight is to breed broad-spectrum disease resistance (*R*) genes into the cultivars. However, potato breeding is complicated because potato cultivars are highly heterozygous autotetraploid plants (2n=4x=4n) and suffer from acute inbreeding depression. On top of that, introduced *R* genes are quickly defeated by "the *R* gene destroyer" *P. infestans*, which has a remarkable capacity to rapidly adapt to resistant plants (Fry 2008; Haas et al. 2009; McDonald and Linde 2002). To minimize the chance of loss of resistance (defeat) of single introduced *R* genes, deploying multiple *R* genes is considered one option for managing the late blight disease (Jones 2001; Park et al. 2009; Pink and Puddephat 1999). Also, quantitative resistance (so-called field resistance) is thought to result in a more durable resistance under field conditions (Fry 2008; Malcolmson 1969; Solomon-Blackburn et al. 2007; Wastie 1991). With late blight resistance breeding having been pretty much unsuccessful for more than 150 years (Müller and Black 1952), breeding for multi-genic resistance that includes field resistance remains a major challenge.

'Sarpo Mira' is one of the few potato cultivars that have been reported to retain resistance in the field for several years and is a candidate for delivering durable late blight resistance (Kim et al. 2011; White and Shaw 2010). The cultivar was bred by the Sárvári family in Eastern Hungary. However, to our knowledge, the pedigree is not described in the literature. Most likely, prebreeding leaned on the heritage of the Russian breeders Vavilov and Bukasov. For the development of other resistant potato cultivars, breeding studies with resistant *Solanum* germplasm have shown that a wealth of *R* genes is present in wild *Solanum* section *Petota* species from South and Central America (Vleeshouwers et al. 2011a). For example, the highly resistant Mexican *Solanum demissum* was used for introgression of *R* genes in potato differential clones *R1 to R11* (Black et al. 1953). Among these, *R1, R2, R3, R4*, and *R10* have been widely used in potato breeding (Vleeshouwers et al. 2011b). Unfortunately, the origin or genetic constituents that determine the resistance of 'Sarpo Mira' are not known, and the degree to which resistance includes previously characterized *R* genes could not be unambiguously determined using classical pathogen assays.

The emerging field of pathogen effector biology has valuable implications for breeding and deployment of disease resistance (Ellis et al. 2009; Vleeshouwers et al. 2011b). Effectoromics involves high-throughput screenings to assign activities to computationally predicted effector genes and has been successful in identifying a growing list of avirulence (*Avr*) effector genes and their matching *R* genes (Oh et al. 2009; Vleeshouwers et al. 2008). To date, more than seven *R*-gene families with distinct functions and effector profiles have been identified and include, among others, *R1, R2, R3a, R3b*, and *R4* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Li et al. 2011; Lokossou et al. 2009; van Poppel et al. 2009). The matching *Avr* genes include *Avr1, Avr2, Avr3a, Avr3b*, and *Avr4* and belong to the large superfamily of host-translocated RXLR effectors of *Phytophthora* spp. (Armstrong et al. 2005; Lokossou et al. 2009; van Poppel et al. 2008) (F. Govers, *per-*

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sonal communication). Knowledge of pathogen effector diversity and mode of action can be utilized to improve the use and deployment of late blight disease resistance to maximize the potential for durability (Vleeshouwers et al. 2011b).

In this study, we pursued an effectoromics approach to unravel the genetic basis of the resistance of 'Sarpo Mira'. First, we generated a population that segregates for resistance to *P. infestans.* We examined the avirulence spectra to different *P. infestans* strains and discovered that multiple *R* genes determine qualitative resistance in 'Sarpo Mira'. Subsequently, we dissected the resistance by functionally testing effectors of *P. infestans* for inducing cell death responses in 'Sarpo Mira'. We identified multiple effectors, including two novel avirulence (AVR) effector proteins that induce specific patterns of responses, each matching a specific *R* gene. Correlating responses to effectors with quantitative resistance in the field indicated that one of the novel *R* genes seems to be responsible for field resistance. These data show that 'Sarpo Mira' contains a pyramid of at least five different *R* genes that confer both qualitative and quantitative resistance to late blight.

RESULTS

Sarpo Mira has complex genetic resistance based on multiple *R* genes.

To dissect the genetic resistance of 'Sarpo Mira', we crossed the resistant 'Sarpo Mira' with the universally susceptible potato breeding clone 'RH89-039-16' (RH), a donor of the recently annotated potato genome (Potato Genome Sequencing Consortium 2011). A subset of 18 genotypes of the resultant 'Sarpo Mira' × RH population (3079) was subjected to disease tests by inoculating detached leaves with a panel of nine diverse *P. infestans* strains, which contain different race structures and are generally complex (Table 1). Qualitative resistance phenotypes were macroscopically scored. Resistant and susceptible phenotypes were observed as a hypersensitive response (HR) or as expanding, sporulating lesions, respectively. Distinct patterns of avirulence were detected among the nine *P. infestans* strains (Table 2). One strain, 89148-09, was avirulent on all offspring plants (pattern A). Three other strains (i.e., PIC99177, EC1, and 90128) were avirulent on at least the offspring plants 3079-4, 3079-6, 3079-7, 3079-8, 3079-16, 3079-27, 3079-28, 3079-31, 3079-37, and 3079-46 (pattern B). Two other distinct resistance patterns were observed for strain H30P04 (pattern C) and PIC99189 (pattern D). The strains PIC99183 and IPO428-2 were able to infect the entire offspring but not the 'Sarpo Mira' parent. In contrast, strain IPO-C was able to fully infect the 'Sarpo Mira' parent as well as the entire 3079 progeny under these test conditions. In some cases, intermediate levels of resistance were observed, which either could be attributed to remarkably high aggressiveness of a strain, such as PIC99183 (Champouret et al. 2009), or could point to the occurrence of other, partial resistance factors that were not further distinguished in these assays. Altogether, the observed distinct resistance patterns observed in these detached-leaf tests indicate that the resistance in 'Sarpo Mira' is genetically highly complex and is composed of several specific *R* genes that are segregating in the 3079 population.

Field resistance is segregating in the 'Sarpo Mira' population.

Although *P. infestans* IPO-C could fully infect 'Sarpo Mira' under detached-leaf test conditions, we became aware that 'Sarpo Mira' does carry a certain level of resistance to this strain in the field. To test whether we could also dissect this field resistance, we planted 30 offspring of the 3079 population, including the same genotypes as were tested on leaves, in the field. In duplicated field trials in 2010 and 2011, potato plants were spray inoculated with strain IPO-C. For determining the quantitative levels of field resistance, we scored the percentages of infected plant tissue in time between 8 and 20 days postinoculation (dpi) and calculated the relative area

Table 1. *Phytophthora infestans* strains used in this study

Strain	Race[a]	Origin	Note or reference
89148-09	0	The Netherlands	Champouret et al. 2009
PIC99177	1.2.3.4.7.9.11	Mexico	Flier et al. 2002
EC1	1.3.4.7.11	Ecuador	Armstrong et al. 2005
90128	1.3.4.7.8.11	The Netherlands	van West et al. 1998
H30P04	3a.7	Lab hybrid	Sibling of T30-4, Haas et al. 2009
PIC99189	1.2.5.7.10.11	Mexico	Flier et al. 2002
PIC99183	1.3.4.5.7.8.10.11	Mexico	Flier et al. 2002
IPO-428-2	1.3.4.7.8.10.11	The Netherlands	Flier et al. 2003
IPO-C	1.2.3.4.5.6.7.10.11	Belgium	Champouret et al. 2009

[a] Race nomenclature is based on ability to infect the differential set of potato carrying *R1 to R11* (Black et al. 1953).

Table 2. *Phytophthora infestans* strains shows distinct patterns of avirulence on a segregating population from 'Sarpo Mira'[a]

	Virulence or avirulence phenotype																				
	Cultivar		Offspring 3079																		
Parents	Sarpo Mira	RH	2	4	6	7	8	10	16	21	24	27	28	31	34	36	37	42	46	48	Pattern
89148-09	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	A
PIC99177	R	S	R	R	R	R	R	R	R	S	S	R	R	R	M	M	R	M	R	M	B
EC1	R	S	M	R	R	R	R	M	R	M	S	R	R	R	S	S	R	S	R	S	B
90128	R	S	S	R	M	R	R	S	R	S	S	R	R	R	S	S	R	S	M	S	B
H30P04	R	S	R	S	R	S	M	R	R	S	M	R	R	R	R	S	R	S	R	S	C
PIC99189	R	S	R	S	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	D
PIC99183	M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	…
IPO428-2	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	…
IPO-C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	…

[a] A detached-leaf assay was performed on the parents (Sarpo Mira and 'RH89-039-16' [RH]) and offspring of population 3079 to score for qualitative resistance to various strains of *P. infestans*. Resistant (R), susceptible (S), and intermediate (M) phenotypes were observed. Various resistance patterns (A to D) were distinguished.

under the disease progress curve (AUDPC) values (Fig. 1). 'Sarpo Mira' remained fully resistant for at least 14 dpi and only 4% of the plant tissue was blighted at 20 dpi. This indicates that, indeed, a certain level of field resistance to strain IPO-C is present in this potato cultivar. The susceptible parent RH was almost fully (98%) diseased at 20 dpi. The offspring plants of 3079 showed a range of intermediate levels of resistance between both parents, in line with the theory that field resistance is typically controlled by so-called minor genes that confer quantitative phenotypes (Gebhardt and Valkonen 2001). In both years, similar results were obtained. In summary, we conclude that field resistance to strain IPO-C segregates in the 'Sarpo Mira' population 3079 even though these plants are fully susceptible to this strain in detached-leaf assays.

Effectoromics screens to dissect late blight resistance in 'Sarpo Mira'.

Effectoromics, or high-throughput screens of plants for response to pathogen effectors, has emerged as a powerful approach to identify and define resistance specificities in *Solanum* spp. germplasm (Vleeshouwers et al. 2008). To test whether the complex resistance of 'Sarpo Mira' could be further dissected using effectors in addition to *P. infestans* strains, we performed an effector screen with an exhaustive collection of 234 predicted RXLR effectors selected from the *P. infestans* genome sequence (Haas et al. 2009). We transiently expressed the effectors in leaves of 'Sarpo Mira' plants by agroinfiltration, in nine replicates. The development of cell death symptoms was visually scored on a scale from 0 to 100%. Of the 234 tested effectors, we found that 30 effectors triggered various levels of cell death in 'Sarpo Mira' (Table 3).

To examine whether responses to effectors co-segregate with specific resistances, we selected 19 effectors that showed the highest levels of cell death (>85%) in 'Sarpo Mira' and used agroinfiltration to express them in population 3079 plants. Subsequently, we scored the plants for occurrence of cell death responses and looked for patterns of co-segregation with specific resistance to the *P. infestans* strains (Table 4). Three effector recognition patterns matched the avirulence profiles of *P. infestans* strains; namely, the responses caused by AVR3b (pattern A), PITG_07550 (pattern B), and AVR4 (pattern C).

Pattern A was found for 14 effectors, which caused cell death in the entire tested population. Among these is *Avr3b* (PITG_18215), the matching *Avr* gene to *R3b* (Li et al. 2011). This suggests that *R3b*, or an *R* gene with *R3b* specificity, is present in 'Sarpo Mira'. Indeed, strain 89148-09, a race 0 that is avirulent on *R3b* potato (Table 1), is also avirulent on 'Sarpo Mira' and all tested 3079 progeny plants (Table 2).

Pattern B matches the response to PITG_07550 and corresponds to the specific resistance to *P. infestans* strains PIC99177, EC1, and 90128. This pattern of responses does not match any of the known *R* genes and, therefore, indicates presence of an as-yet-unknown *R* gene in 'Sarpo Mira'. We designated this gene *Rpi-Smira1*, and the matching PITG_07750 as the candidate for *AvrSmira1*.

Pattern C reflects the response to AVR4 (PITG_07387) and specific resistance to H30P04. Indeed, strain H30P04 is avirulent on *R4* potato (Table 1). The data suggest that *R4,* or an *R* gene with *R4* specificity, is present in 'Sarpo Mira' and segregating in population 3079.

In addition to these three patterns that clearly match response to single effectors (patterns A to C), three other response patterns (patterns D to F) could be distinguished using effector assays. In case of responses to AVR3a (pattern F) and AVR4 (pattern C), their combined pattern (D) matches the avirulence profile to strain PIC99189. These data suggest that this strain has both *Avr3a* and *Avr4*, which is also in agreement with its race structure (Table 1). In line with this, the presence of *Avr3a* and *Avr4* coding genes was predicted in the genome of PIC99189 genome by alignment of Illumina reads of PIC99189 to the reference genome strain T304 (Raffaele et al. 2010) (L. Cano, and S. Kamoun, *unpublished*).

To further examine the correlation between effector response and resistance patterns, we performed an analysis of variance (ANOVA) on results from the detached-leaf tests and agroinfiltrations (Supplementary Table S1). Patterns B, C, and D were, indeed, statistically supported (P values < 0.0001). Pattern A could not be tested because response to AVR3b did not segregate in the population.

In summary, effectoromics revealed additional patterns of response to *P. infestans* and four effectors that are recognized (AVR3a, AVR3b, AVR4, and AVRSmira1) to match avirulence patterns of *P. infestans* strains (Table 4). We summarize the key features these four *Avr* genes of *P. infestans* in Figure 2, based on the genome data of the reference strain T30-4 (Haas et al. 2009): i) the modular structure that is typical for RXLR effectors with an N-terminal (signal peptide) domain, RXLR motif, and C-terminal effector domain; ii) expression of the *Avr* genes is induced during the early biotrophic phase of the infection; and iii) the *Avr* genes occur in the gene-sparse regions of the genome. The new *AvrSmira1* fits all these criteria that are typical for all *Avr* genes of *P. infestans* that have been identified thus far (Vleeshouwers et al. 2011b).

Response to PITG_07558 correlates with field resistance and reveals a fifth resistance gene, *Rpi-Smira2*, that confers quantitative late blight resistance.

Responses to the remaining effector, PITG_07558, did not match the resistance profiles of any of the examined *P. infestans* strains that were assayed on detached leaves. Therefore, we decided to test whether these responses matched the observed field resistance in 'Sarpo Mira' (Table 2; Fig. 1). An

Fig. 1. Photographs of potato in the field trail of 2010 at 34 days postinoculation. **A,** Susceptible parent 'RH89-039-16'; two partially resistant offspring plants **B,** 3079-16 and **C,** 3079-48; and **D,** the field resistant 'Sarpo Mira'.

ANOVA analysis on the average AUDPC values of both field trials and responses to the effectors revealed that responses to PITG_07558 significantly correlated with field resistance, especially during the first 3 weeks after infection ($P < 0.0025$) (Fig. 3). These data show that recognition of one effector, PITG_07558, co-segregates with field resistance, conferred by a fifth gene we named *Rpi-Smira2*. We designated PITG_07558 as the candidate *AvrSmira2* gene. Similar to the other four *Avr* genes, *AvrSmira2* occurs in the gene-sparse regions of the genome and has the modular structure that is typical for RXLR effectors, with an N-terminal (signal peptide) domain, RXLR motif, and C-terminal effector domain (Fig. 2). However, compared with the other *Avr* genes, expression of *AvrSmira2* is induced at earlier time points (i.e., 16 versus 48 h postinoculation [hpi]). Despite this shift in timing of upregulation, *AvrSmira2* is expressed during early biotrophic phases, which can lead to recognition by plant receptors and initiation of avirulence activity.

Table 3. RXLR effectors that trigger cell death response on 'Sarpo Mira'[a]

Effector		**SignalP[b]**				**Expression in potato[c]**		**Response**	
Gene or construct ID	**Gene annotation**	**HMM prob.**	**NN mean NS score**	**RXLR**	**RXLR tribe[d]**	**16 hpi**	**48 hpi**	**'Sarpo Mira' (%)[e]**	**Pattern in 3079[f]**
PITG_12761	…	0.999	0.791	RLLR	1	0.73	0.04	31	na
PITG_15039	…	0.993	0.928	RILV	1	0.83	1.12	100	Pattern A
PITG_22880	…	0.999	0.928	na	1	0.74	0.09	100	Pattern A
PITG_07558	*AvrSmira2*	1.000	0.963	RSLR	2	1.11	0.24	100	Pattern E
PITG_09660	…	0.999	0.948	na	2	1.38	0.81	94	Pattern G
PITG_04085	…	1.000	0.879	RSLR	5	–0.10	2.53	92	Pattern A
PITG_04090	…	1.000	0.849	RSLR	5	–0.62	2.44	36	na
PITG_04097	…	1.000	0.786	RSLR	5	1.40	1.28	92	Pattern A
PITG_18683	…	1.000	0.862	RSLR	5	–0.54	2.61	89	Pattern A
PITG_20300	…	1.000	0.849	RSLR	5	–1.13	2.45	83	Pattern A
PITG_20301	…	1.000	0.889	RSLR	5	–0.12	2.39	100	Pattern A
PITG_20303	…	1.000	0.880	RSLR	5	0.16	2.39	90	Pattern A
PITG_04169	…	0.995	0.918	RSLR	10	0.13	0.65	92	Pattern A
PITG_15718	…	0.994	0.754	RSLR	14	1.72	0.45	22	na
PITG_05750	…	1.000	0.934	RLLR	29	1.52	1.04	33	na
PITG_16275	…	0.999	0.869	RLLS	42	0.75	2.55	11	na
PITG_15162	…	0.999	0.903	RRLR	43	1.74	0.58	22	na
PITG_07387	*Avr4*	0.999	0.850	RFLR	52	0.03	2.00	100	Pattern C
PITG_10540	…	0.997	0.747	RFLR	57	–0.06	1.37	85	Pattern A
PITG_14371	*Avr3a* (EM)	na	na	RLLR	58	0.72	2.20	na	na
PITG_14371_KI[g]	*Avr3a* (KI)	na	na	RLLR	58	na	na	100	Pattern F
PITG_14374	…	0.992	0.724	RFLR	58	0.44	0.91	100	Pattern A
PITG_04049	…	0.997	0.744	QFLR	67	0.49	2.47	14	na
PITG_14360	…	0.999	0.953	RILR	72	0.97	0.93	21	na
PITG_16294	…	1.000	0.933	RLLR	97	0.51	2.63	6	na
PITG_07550	*AvrSmira1*	1.000	0.947	RLLR	117	1.66	2.24	100	Pattern B
PITG_18215	*Avr3b*	0.999	0.857	RSLR	124	1.51	3.07	100	Pattern A
PITG_23129	…	0.956	0.452	RLLR	128	1.38	1.28	72	Pattern A
PITG_23131	…	0.983	0.831	RLLR	128	0.97	1.09	100	Pattern A
PITG_19942	…	0.998	0.948	RYLK	237	0.62	1.23	34	na
pMDC32	Neg control	…	…	…	…	na	na	0	None
R3a:*Avr3a*	Pos control	…	…	…	…	na	na	100	Pattern A

[a] Description of predicted RXLR effectors from the *Phytophthora infestans* genome reference strain T30-4.
[b] Hidden Markov model (HMM) score and S-mean value predicted using SignalPv2.0. NN = neural networks output, NS = S score (signal peptide score).
[c] Expression in reference genome T30-4, relative expression levels during infection compared with mycelium; hpi = hours postinoculation.
[d] Tribe numbers as described by Haas and associates (2009).
[e] Percentage of cell death response upon agroinfiltration, based on an average of quantitative scores in at least nine replicates.
[f] Segregation pattern in 3079; na = not assessed
[g] Avr3aKI is the avirulent allele of Avr3aEM, which is represented in the reference genome strain T30-4.

Table 4. Segregation of response to RXLR effectors in 20 offspring genotypes of the 'Sarpo Mira' (SM) × 'RH89-039-16' (RH) population 3079[a]

		Response in plants																				
Effector		**Parents**		**Offspring of 3079**																		
Gene, construct ID	**Annotation[b]**	**SM**	**RH**	**2**	**4**	**6**	**7**	**8**	**10**	**16**	**21**	**24**	**27**	**28**	**31**	**34**	**36**	**37**	**42**	**46**	**48**	**Pattern**
PITG_18215	*Avr3b*	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A
PITG_07750	*AvrSmira1*	+	–	–	+	+	+	+	–	+	–	–	+	+	+	–	–	+	–	+	–	B
PITG_07387	*Avr4*	+	–	+	–	+	–	–	–	+	–	–	+	+	+	+	–	+	–	+	–	C
PITG_07758	*AvrSmira2*	+	–	+	+	–	–	–	+	+	+	–	+	+	+	–	+	–	–	–	–	E
PITG_14371	*Avr3a*	+	–	+	–	+	–	–	+	+	+	+	–	–	+	+	+	–	+	–	+	F
PITG_14371 + PITG_07387	*Avr3a + Avr4*	+	–	+	–	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	D
pK7WG2	Neg control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G
R3a & Avr3a	Pos control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A

[a] Effectors expressed in pK7WG2 were functionally tested in leaves of parents and offspring plants and monitored for occurrence (+) or absence (–) of cell death responses.
[b] Gene annotation.

***AvrSmira1* has diverse genetic variants with similar specificity.**

AvrSmira1 (PITG_07550) originates from the *P. infestans* reference strain T30-4. To detect additional variants of *AvrSmira1*, we performed a polymerase chain reaction survey on the 9 *P. infestans* strains (Table 1). We detected a total of 22 single-nucleotide proteins (SNP) when comparing the homologous coding sequences of PITG_07550. Of these, 20 SNP are found in the C-terminal effector domain of PITG_07550 (the signal peptide was not analyzed). Of 20 C-terminal SNP, 14 represent nonsynonymous substitutions that lead to 13 polymorphic amino acid residues at the C-terminal effector domain of PITG_07550. In total, we identified 12 different AVRSmira1 amino acid variants (Fig. 4A).

Phylogenetic analyses of the protein alignment of the 12 AVRSmira1 variants using maximum parsimony algorithms showed a grouping into two broad classes, I and II (Fig. 4B). Phylogenetic trees based on neighbor-joining or minimal evolution resulted in similar clustering (not shown).

To investigate whether AvrSmira1 is also under diversifying selection, we assessed positive selection per residue on the obtained AvrSmira1 variants. With model M1, M2, and M8, eight or nine amino acids were identified, of which the eight were always overlapping. Among those eight, four amino acids on positions 128, 130, 156, and 170 were significantly under diversifying selection (Fig. 4A; Supplementary Table S2).

To study whether the genetic variants of AVRSmira1 in *P. infestans* strains can explain avirulence properties on *Rpi-Smira1*-containing potato, we compared their phylogenetic clustering and significant amino acids with resistance profiles obtained from progeny plants (Tables 5 and 6). Most *P. infestans* strains are able to infect one or more progeny genotypes that respond to the AVRSmira1 candidate, which indicates that *Rpi-Smira1* is defeated by most tested strains. However, the strains EC1 and PIC99177 are most likely avirulent on *Rpi-Smira1* plants because they were not able to infect any of the AVRSmira1-responding progeny plants (Table 2). These strains lack class I AVRSmira1 variants, and lack a methionine or arginine on amino acids 156 and 170, respectively (Fig. 4A). This suggests that class II variants are potentially sufficient to mediate avirulence on *Rpi-Smira1* plants. To test whether genetic variants can induce cell death on 'Sarpo Mira', we expressed variants 1, 4, 5, 11, 8, 9, 10, 14, and 15 in *Agrobacterium* spp. and tested them by agroinfiltration in two plants. All variants were able to induce cell death; however, variant PITG_07550_10 seemed to induce slightly lower levels of cell death (data not shown). Taken together, our results show that AVRSmira1 is a highly polymorphic gene that is under positive selection in *P. infestans*. It remains to be determined which characteristics of this gene are important for its avirulence activity on 'Sarpo Mira'.

DISCUSSION

The potato 'Sarpo Mira' has been proposed to carry valuable and potentially durable resistance to *P. infestans* (Kim et al. 2011; White and Shaw 2010). In this study, we show that the genetic basis of late blight resistance in 'Sarpo Mira' is highly complex, consisting of at least five different *R* genes that confer qualitative and quantitative resistance to late blight. We dissected this multifaceted resistance using effector assays and a differential set of *P. infestans* strains. Profiling the effector responses enabled a better discrimination of specific *R* gene activities than pathogen assays with a collection of *P. infestans* strains. This study vividly illustrates the value of integrating effector screens (effectoromics) with the genetic characterization of plant breeding material (Vleeshouwers et al. 2011b). With these five effectors at hand, breeding of 'Sarpo Mira'–derived potato can proceed in a more precise and efficient fashion.

Our effectoromics approach enabled the dissection of late blight resistance in 'Sarpo Mira' into distinct components. First, the qualitative *R* genes *R3a, R3b*, and *R4* could be accurately identified and profiled using *Avr3a, Avr3b*, and *Avr4*, independently of other *R* genes. This could not be performed solely using differential *P. infestans* strains, because multiple *R* genes independently segregate in the progeny and can mask other recognition specificities. Second, we discovered novel *R*

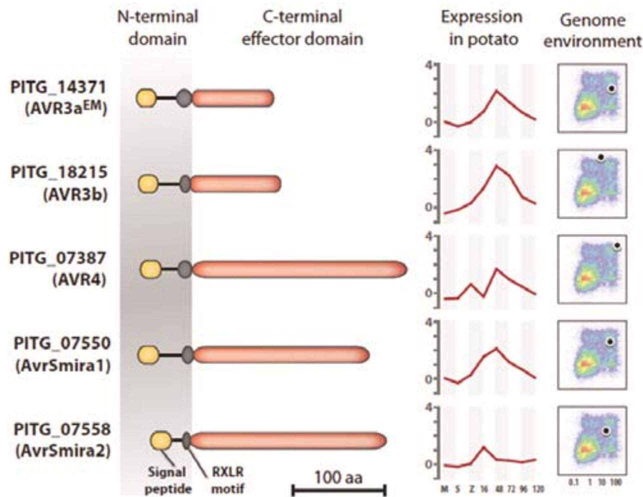

Fig. 2. Features of the five effectors that have avirulence (AVR) activity on 'Sarpo Mira'. The figure depicts AVR3a, AVR3b, AVR4, AVRSMira1, and AVRSMira2. The domain structure of *Phytophthora infestans* AVR proteins shows a typical RXLR effector modular structure with N-terminal (signal peptide) domain, RXLR motif, and C-terminal effector domain. Graphs representing the expression in potato illustrate a time course expression pattern of the *Avr* genes during 16 to 120 h of infection of potato by genome reference strain T30-4, with the *y* axis showing gene induction (Haas et al. 2009). Genome environment heat maps are two-dimensional plots of 5′ and 3′ intergenic distance for all *P. infestans* genes (Haas et al. 2009).

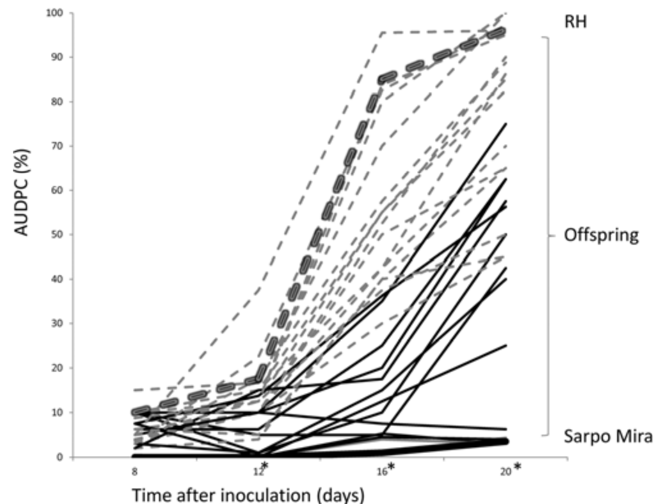

Fig. 3. Quantitative field resistance of 'Sarpo Mira' to virulent *Phytophthora infestans* IPO-C. Levels of late blight infection for 'Sarpo Mira', 'RH89-039-16' (RH), and their offspring are plotted from 8 to 20 days postinoculation (dpi) from the field trail in 2011. 'Sarpo Mira' and partially resistant offspring genotypes showed cell death to PITG_07558 (solid lines), whereas RH and remaining offspring genotypes failed to recognize the effector (dashed lines). Significance of correlation between response to PITG_07558 and field resistance is indicated (*) for each time point at the *x* axis for *P* values lower than 0.001.

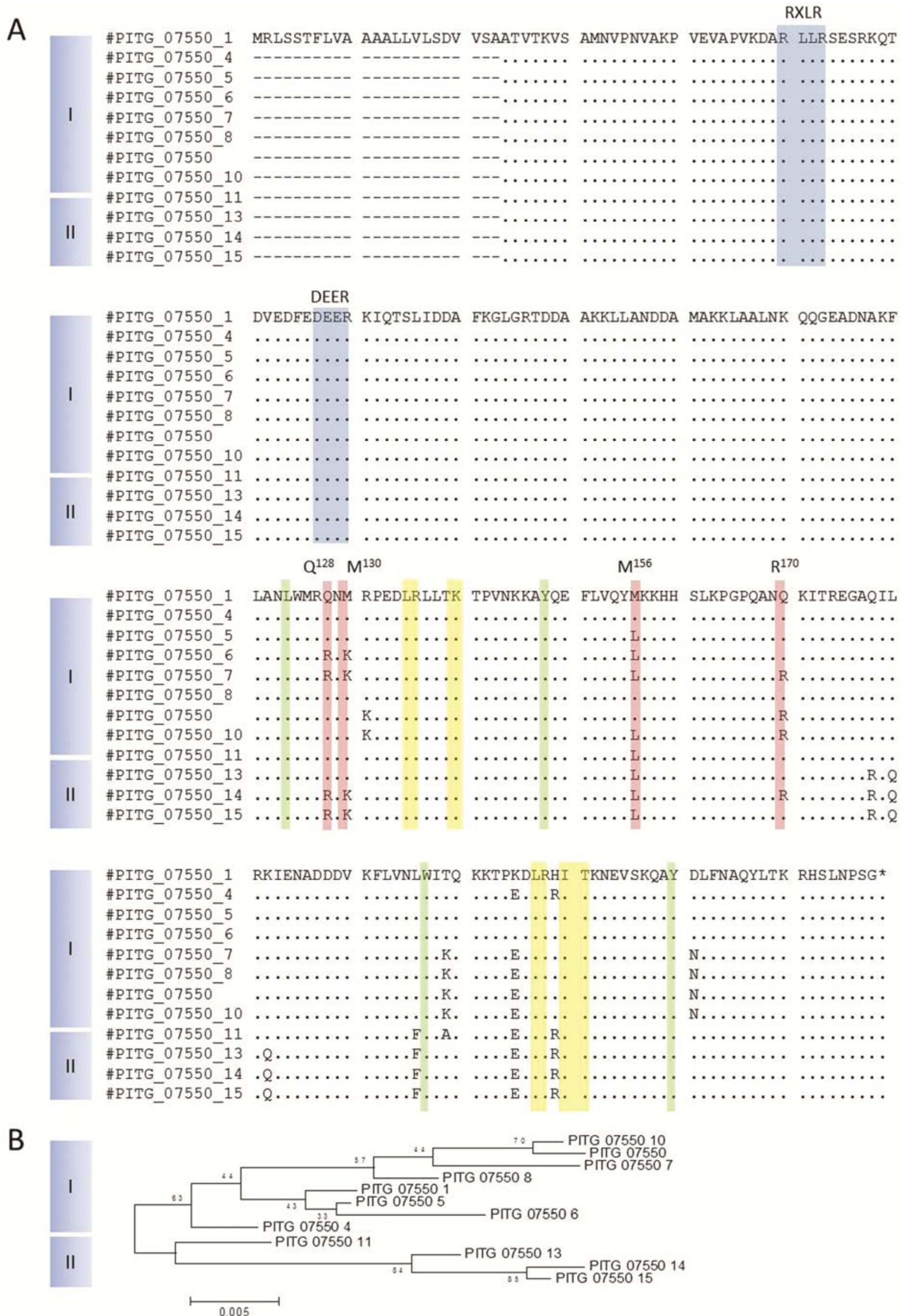

Fig. 4. Genetic variants of the AVRSmira1 candidate PITG_07550 in *Phytophthora infestans* strains. **A,** Alignment of amino acid sequences of AVRSmira1 variants, after the signal peptide. The RXLR-DEER motifs are marked in gray, amino acids under diversifying selection in red, and two putative WY domains in yellow and green. **B,** Neighbor-joining tree reveals two classes of AVRSmira1 variants. Bootstrap values of 1,000 replicates are indicated at the nodes.

genes based on unique response profiles to two novel avirulence effector candidates, AVRSmira1 and AVRSmira2. This would have been much more challenging using *P. infestans* strains, especially given that these two *R* genes co-occur with three other resistance specificities. Third, in addition to detecting qualitative *R* genes such as *R3a, R3b, R4*, and *Rpi-Smira1,* the effector assays unraveled *Rpi-Smira2*, a gene that confers quantitative field resistance. Because partial resistance conferred by *Rpi-Smira2* is often overshadowed by *R* genes with stronger effects, its detection is challenging, if not impossible, with *P. infestans* strains alone. In this case, the *Rpi-Smira2* partial resistance could only be scored in field trials and not in laboratory assays. One challenge for scoring such a type of resistance is that field trials are vulnerable to cross infections by airborne strains with unknown specificities, thereby greatly complicating the phenotyping. With the discovery of the AVRSmira2 effector, we now have a straightforward and precise assay for *Rpi-Smira2*, which could accelerate and facilitate the breeding and cloning of this gene.

All five RXLR effector genes fulfill the typical features of oomycete *Avr* genes as they encode for modular proteins that contain an N-terminal signal peptide followed by a RXLR motif and a C-terminal effector domain, occur in gene-sparse regions of the *P. infestans* genome, and are induced during the early biotrophic phases of infection (Fig. 2). Avirulence activities of AVR3a, AVR3b, and AVR4 correspond to the race profiles, the presence or absence in genome sequence of T30-4 and PIC99189, and published genotype–phenotype relationships (Armstrong et al. 2005; Li et al. 2011; van der Lee et al. 2001; van Poppel et al. 2008). The newly identified *AvrSmira1* is induced during the biotrophic phases of infection in *P. infestans* based on microarray gene expression data. Also, a partial sequence of *AvrSmira1* is present in an expressed sequence tag library from germinating cysts of *P. infestans* (GenBank accession CV920103). Small-scale genetic variation studies confirmed that AVRSMira1 is polymorphic within nine analyzed *P. infestans* strains, similar to some other AVR proteins (e.g., AVRblb1 of *P. infestans*) (Champouret et al. 2009; Halterman et al. 2010). We identified up to 12 different variants of AVRSmira1 that share 13 polymorphic amino acids. These polymorphisms mainly occurred at the C-terminal effector domain, which is known to be involved in recognition specificity (Win et al. 2007). Correlating the genetic variants with virulence on *Rpi-Smira1* plants revealed the absence of class I variants carrying the M^{156} and R^{170} polymorphisms in avirulent strains, indicating that these genes are not essential for avirulence. The amino acids found under diversifying selection and, for example, a weaker degree of recognition or lower expression levels of the effector genes in virulent isolates could play a role in determining avirulence (Oh et al. 2009; Qutob et al. 2009).

The other newly identified *Avr* candidate *AvrSmira2* was induced only at 16 hpi during a time course of infection on potato (Fig. 2). This is earlier than the timing for gene induction from other known avirulence effector genes of *P. infestans*, which peak at 48 hpi (Haas et al. 2009; Vleeshouwers et al. 2011b) (Fig. 2). This transient and early induction profile of *AvrSmira2* could explain why its cognate *R* gene, *Rpi-Smira2*, only confers quantitative resistance. The extent to which expression profiles, genetic variation, or other factors explain the recognition of this effector in relation to field resistance remains to be investigated.

Field resistance to late blight has been claimed to be durable in various studies (Fry 2008; Stewart et al. 2003; Van Der Plank 1971; Wastie 1991). Given that *R* genes are typically quickly defeated because of the ability of *P. infestans* to rapidly adapt and evolve (Raffaele et al. 2010; Wastie 1991), many potato breeders were caught by naïve optimism that breeding for "field resistance" should prove more durable. This has led to the misguided concept of breeding for so-called "*R*-gene-free" potato plants that carry field resistance but lack known *R* genes. However, the genetic basis of field resistance has remained unclear, mainly because the weak phenotypes are too difficult to follow in the genetically complex potato and

Table 5. Overview of genetic diversity of AVRSmira1 in relation to virulence on the 'Sarpo Mira' × 'RH89-039-16' population 3079[a]

		Positive selected amino acid sites				*Phytophthora infestans* strains								
Variant	**Class**	**128**	**130**	**156**	**170**	**89148-09**	**PIC 99177**	**EC1**	**90128**	**H30P04**	**PIC 99189**	**PIC 99183**	**IPO 428-2**	**IPO-C**
PITG_07550[b]	I	Q	K	M	R	…	…	…	…	…	…	…	…	…
PITG_07550_1	I	Q	K	M	Q	X	…	…	…	X	…	…	X	X
PITG_07550_4	I	Q	K	M	Q	X	…	…	…	…	…	…	…	…
PITG_07550_5	I	Q	K	L	Q	…	…	…	…	…	…	X	…	…
PITG_07550_6	I	R	M	L	Q	…	…	…	…	…	…	…	X	…
PITG_07550_7	I	R	M	L	R	…	…	…	X	…	…	…	…	…
PITG_07550_8	I	Q	K	M	Q	…	…	…	…	X	…	…	…	…
PITG_07550_10	I	Q	K	L	R	…	…	…	X	X	…	…	…	…
PITG_07550_11	II	Q	M	L	Q	…	X	…	…	…	X	…	…	…
PITG_07550_13	II	Q	M	L	Q	…	…	…	…	…	…	X	…	…
PITG_07550_14	II	R	K	L	R	X	…	…	…	…	…	X	…	…
PITG_07550_15	II	R	K	L	Q	X	X	X	X	…	X	X	X	X

[a] Genetic diversity of AvrSmira1 in *P. infestans* strains. For each obtained genetic variant, the phylogenetic classification, identity of amino acids under diversifying selection, and presence in *P. infestans* strains is presented.
[b] Broad PITG_07550 sequence.

Table 6. Summary of virulence data on the 3079 population in offspring that was responding versus not responding to the *AvrSmira1* candidate PITG_07550[a]

	Phytophthora infestans strains								
Population 3079	**89148-09**	**PIC 99177**	**EC1**	**90128**	**H30P04**	**PIC 99189**	**PIC 99183**	**IPO 428-2**	**IPO-C**
Response	R	R	R	R-S	R-M-S	R-S	S	S	S
No response	R	R-M-S	M-S	S	R-M-S	R	S	S	S

[a] Individual plants were scored resistant (R), intermediate (M), or susceptible (S).

because the AVR profiles of infecting *P. infestans* strains could not accurately be determined. In this study, we took advantage of the *P. infestans* genome sequence and the complete catalogue of RXLR effector genes to successfully apply an effectoromics strategy. We showed that field resistance can be dissected and assessed using pathogen effector assays. The response to AVRSmira2 was significantly correlated with quantitative *Rpi-Smira2* resistance in the field. This indicates that effectors can serve as absolute markers in field resistance breeding programs. In addition, our results suggest that field resistance shares mechanistic similarities with *R*-gene-based resistance, and might also be mediated by nucleotide-binding site leucine-rich repeat (NBS-LRR)-type *R* genes and perception of RXLR effectors. This is in line with reports that field resistance loci are localized in regions of the potato genome that contain NBS-LRR genes (Leonards-Schippers et al. 1994; Tan et al. 2008) and that potato plants with field and partial resistance mount the HR after penetration of *P. infestans* (Gees and Hohl 1988; Kamoun et al. 1999; Vleeshouwers et al. 2000). Therefore, field resistance may represent a weaker form of *R*-gene-based resistance that does not cause a full-blown HR and resistance phenotype upon recognition of the pathogen. Our findings that field resistance of ‘Sarpo Mira’ is associated with recognition of an RXLR effector opens up new possibilities to further unravel the underlying resistance mechanism.

The newly identified qualitative *R* gene, *Rpi-Smira1,* can be exploited in potato breeding. Based on a disomic segregation model and because recognition of AVR3a is in repulsion phase with AVRSmira1-mediated resistance, we hypothesize that *Rpi-Smira1* most likely localizes near the *R3a* gene cluster on chromosome 11 on a sister chromatid.

In modern late blight management, breeders attempt to preempt the quick defeat of *R* genes by pyramiding multiple *R* genes in one cultivar (Jacobsen and Hutten 2006; Pink and Puddephat 1999). In ‘Sarpo Mira’, the previously defeated *R3a, R3b*, and *R4* genes are present and confer full qualitative resistance to avirulent strains in detached-leaf tests, similar to the other qualitative *R* genes *R1*, *R2*, and *R3a* that have been isolated from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009). In addition to *R3a, R3b*, and *R4*, a new race-specific *R* gene, *Rpi-Smira1*, was identified in ‘Sarpo Mira’. Like the other qualitative *R* genes from *S. demissum, Rpi-Smira1* was also defeated because strains H30P04, PIC99189, PIC99183, IPO428-2, and IPO-C can fully infect progeny plants that contain *Rpi-Smira1*. However, from a practical perspective, it should be kept in mind that even such defeated *R* genes may cause a delay in infection, as reported, for instance, for the *Solanum R2* gene (Pilet et al. 2005). In addition, the degree of resistance is dependent on the genotype of the *P. infestans* population, and monitoring for virulence to specific *R* genes in the local *P. infestans* population can assist *R* gene deployment (Champouret et al. 2009; Vleeshouwers et al. 2011b).

Sarpo Mira has performed better in the field and has been proposed to be the answer to potato late blight resistance compared with other potato cultivars with combined *R* genes, such as ‘Pentland Dell’ (*R1R2R3*) and ‘Escort’ (*R1R2R3R10*). Our study clearly demonstrates that ‘Sarpo Mira’ contains a pyramid of not only four qualitative *R* genes but also one quantitative field resistance gene. Therefore, late blight resistance of ‘Sarpo Mira’ may simply be due to a combination of *R*-gene-type responses to a set of five RXLR effectors, and evolution of virulence in *P. infestans* may be dependent on epidemiological factors, such as acreage and the pathogen races present in the field. Indeed, our work shows that some isolates, such as IPO-C, are virulent on ‘Sarpo Mira’ in the lab and in the field. Nonetheless, we now have the molecular markers and effector assays to breed the five resistance genes and improve the agronomic traits of ‘Sarpo Mira’. In addition, in the future, the approach of combining qualitative *R* genes with quantitative ones should be given more consideration in late blight resistance breeding.

MATERIALS AND METHODS

Plant material.

The potato ‘Sarpo Mira’ was crossed with the susceptible RH, a donor of the potato genome sequence (Potato Genome Sequencing Consortium 2011). Thirty genotypes of the resulting ‘Sarpo Mira’ × RH population 3079, and parents, were clonally maintained in vitro in sterile jars containing Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 3% (wt/vol) sucrose at 20°C.

P. infestans strains and disease tests.

P. infestans strains were grown on rye agar medium supplemented with 2% (wt/vol) sucrose (Caten and Jinks 1968) and incubated at 15°C in the dark. A list of *P. infestans* strains used in this study is presented in Table 1. For inoculation of detached leaves, a plate covered with mycelium was flooded with water and the sporangiophore suspension was pipetted into a test tube. After 1 to 2 h of incubation at 4°C, zoospores were released and a spore suspension of 5×10^4 spores/ml was prepared.

Detached-leaf tests were performed as previously described, with slight modifications (Vleeshouwers et al. 1999). Leaves were detached from mature greenhouse-grown plants, placed in water-saturated florists foam (Oasis) in a tray, and spot inoculated by pipetting 10-µl droplets of the spore suspension on the abaxial side. The trays were covered with transparent bags and incubated in a climate chamber at a photoperiod 16 and 8 h (day and night, respectively) at 15°C. At 6 dpi, disease phenotypes on the leaves were macroscopically scored. Leaves displaying no disease symptoms or small and localized HR lesions were scored as resistant, whereas leaves containing expanding lesions with massive sporulation were scored susceptible. In some cases, intermediate symptoms occurred, representing less-expanded lesions without or with very limited sporulation. For each *P. infestans* strains, at least five inoculation spots were performed on at least two leaves, resulting in 10 replicates per experiment. All strains were tested twice in independent experiments, except IPO-C, which was tested in a single experiment.

Field trials were performed in summer 2010 and 2011 in Wageningen, The Netherlands. ‘Sarpo Mira’ parents and progeny genotypes were represented as two-plant plots, which were treated as single experimental units as described by Colon and Budding (1988). Spreader rows consisted of ‘Bintje’ potato and border rows of ‘Nicola’ potato. At nightfall, a zoospore suspension of IPO-C was sprayed on the potato field using a tractor with two spraying arms. Disease assessments were made by estimating the percentage of leaf area covered with late blight lesions for each plot. From these readings, the AUDPC was calculated (Fry 1978). In 2010, disease assessments were made between 22 and 47 dpi and, in 2011, between 8 and 20 dpi.

Effector screening.

A genome-wide collection of RXLR effectors was selected from the *P. infestans* genome sequence based on presence of a predicted signal peptide, an RXLR motif, and an elevated gene expression at 6 hpi to 3 dpi (Haas et al. 2009). Sequences coding for putative signal peptides were excluded and replaced with an ATG codon. The coding sequences were synthesized by GenScript Inc. (Piscataway, NJ, U.S.A.) in between AttL1

and AttL2 gateway recombination sites and cloned into pUC57. Subsequently, effectors were subcloned into pK7WG2 using LR clonase (Invitrogen, Carlsbad, CA, U.S.A) and were transformed into *Agrobacterium tumefaciens* AGL1, pSoup, and pVirG cells by electroporation.

Agroinfiltration was performed as previously described (van der Hoorn et al. 2000; Vleeshouwers and Rietman 2009). Briefly, *A. tumefaciens* strains were grown to a desired optical density at 600 nm of 0.3 and leaf panels of 2- to 3-week-old potato plants were infiltrated with the *A. tumefaciens* suspensions. Symptoms were monitored from 2 to 6 days postinfiltration.

Genetic variation of AvrSmira1.

AvrSmira1 variants were amplified from genomic DNA of *P. infestans* strains with the forward (GGGGACAAGTTTgtaca aaaaagcaggctATatgGCAACTGTGACCAAGGTATCG) and reverse (GGGGACCACTTTGTACAAGaaagctgggtATTATCCG GAGGGGTTTAGC) primers using Phire and Phusion polymerase (Finnzymes). PITG_7550_1 and PITG_10 were also detected in the T30-4 genome trace archive at the National Center for Biotechnology Information (Robbertse and Tatusova 2011) using BlastN and consequent contig assembly using DNAstar v9. Amplicons were cloned into pK7WG2 and transformed to *A. tumefaciens* AGL1. DNA sequences were analyzed using DNAstar v9. Phylogenetic analyses of *AvrSmira1* sequences were conducted using the minimum evolution method, neighbor joining, and maximum parsimony using MEGA version 5 (Tamura et al. 2011). DNA sequences of the 11 *AvrSmira* variants were subjected to positive selection analysis using the software package JCoDA (Steinway et al. 2010), in which the PAML v.4 codeml program (Yang 2007) was integrated. Predictions for WY domains were performed with the RADAR repeat finder EMBL server.

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The Sárvári Research Trust website: www.sarvari-trust.org

Center for Biological Sequence Analysis SignalPv2.0 server: www.cbs.dtu.dk/services/SignalP-2.0