Table 1. Functional profiling of RXLR effectors in *Solanum*.

Origin	Series according to Jacobs et al. (2008)	*Solanum* species	Genebank[*]	Resistance[†]	Names of Genotypes
				P. infestans 90128	
Bolivia	Circaeifolia Piurana Megistacroloba Tuberosa	*S. capsicibaccatum*	CGN 22388-1	R	cap22388-1
Bolivia	Circaeifolia Piurana Megistacroloba Tuberosa	*S. circaefolium subsp. quimense*	CGN 18158-1	R	qum18158-1
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. polyadenium*	CGN 17749-4	R	pol17749-4
USA	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. jamesii*	CGN 18349-1	R	jam18349-1
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. pinnatisectum*	CGN 17743-4	R	pnt17743-4
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. pinnatisectum*	CGN 23011-1	R	pnt23011-1
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	Blb99-256-3	R	blb99-256-3
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	2002	R	blb2002
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	8005-8	R	blb8005-8
Mexico	Longipedicellata	*S. stoloniferum*	CGN 17605-4	R	sto17605-4
Mexico	Longipedicellata	*S. papita*	CGN 18309-1	R	pat18309-1
Mexico	Longipedicellata	*S. stoloniferum*	CGN 17606-2	R	sto17606-2
Peru	Longipedicellata	*S. huancabambense*	CGN 17719-1	R	hcb17719-1
Mexico	*S. verrucosum*	*S. verrucosum*	CGN 17768-10	R	ver17768-10
Mexico	Demissa Conicibaccata	*S. schenckii*	PI 498042-1	R	snk498042-1
		dihaploid clone from AM78-3778	AM3778-16	R	AM3778-16
Peru	Cultivated Tuberosa and Tuberosa from Peru	*S. machiquense*	GLKS 2319-2	R	mac2319-2
Bolivia	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. avilesii*	CGN 18256-2	R	avl18256-2
Bolivia	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. chacoense*	CGN 962709-5	R	cha962709-5
Bolivia	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 23050-1	R	mcd23050-1
Argentina	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 17596-8	R	mcd17596-8
Bolivia	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 18200-6	R	mcd18200-6
Bolivia	Tuberosa from Bolivia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 21342-1	R	mcd21342-1
Argentina	Tuberosa Megistacroloba	*S. okadae*	CGN 18000-1	R	oka18000-1
		S. tuberosum cv.Impala-*Rpi-blb1*		R	Impala-*Rpi-blb1*
		S. tuberosum cv. Impala		S	Impala
		S. tuberosum cv. Désirée		S	Désirée
		S. tuberosum cv. Bintje		S	Bintje

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Table 1. (*Continued*) Functional profiling of RXLR effectors in *Solanum*.

Names of Genotypes	Specific																											
	1 PexRD8	2	3	4	1 PexRD11	2	1 PexRD1	1 PexRD3	1 PexRD4	1 PexRD10	1 PexRD7	2	3	1 PexRD13	2	1 PexRD14	2	1 PexRD16	2	1 PexRD17	2	1 PexRD21	1 PexRD28	1 PexRD22	2	2 PexRD26	1 PexRD24	2
	ipiO1	ipiO2	ipiO3	ipiO4	21-1	43-1	195-2	12-9	53-1	96-1	Avr3a-KI	Avr3a-EM	Pex147-2	98-3	98-4	99-4	99-5	56-2	59-1	MKA-1	MKB-4	64-2	176-2	68-2	66-1	118-1	113-1	116-1
cap22388-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
qum18158-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
pol17749-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
jam18349-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
pnt17743-4	-	-	nd[2]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pnt23011-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb99-256-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb2002	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb8005-8	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+
sto17605-4	+[1]	+[1]	-	+[5]	-	-	-	-	-	-	-	-	-	+[5]	-	-	-	-	-	+[5]	-	-	+[3]	-	-	+[5]	-	-
pat18309-1	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
sto17606-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
hcb17719-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+
ver17768-10	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
snk498042-1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AM3778-16	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moc2319-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
avl18256-2	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cha962709-5	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
mcd23050-1	-	-	nd	-	-	-	+[3]	-	-	-	+[3]	-	-	-	+[3]	-	-	+[3]	-	-	-	+[3]	-	-	-	-	+[3]	+[3]
mcd17596-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
mcd18200-6	-	-	-	+	-	-	-	+[4]	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
mcd21342-1	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
oka18000-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Impala-*Rpi-blb1*	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Désirée	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bintje	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1. (*Continued*) Functional profiling of RXLR effectors in *Solanum*.

Names of Genotypes	Specific													Non-specific														Controls	
	PexRD31	PexRD36		PexRD41			PexRD45		PexRD46			PexRD50	PexRD51	PexRD2	PexRD7	PexRD8	PexRD12		PexRD26	PexRD27	PexRD39/40				PexRD44	PexRD49	PexRD50	neg	pos
	1	1	2	1	2	3	1	2	1	2	3	1	1	1	4	1	1	2	1	1	1	2	3	4	1	1	2		
	120-1	45-1	45-10	91-3	91-5	91-7	184-2	215-3	92-4	92-2	92-12	191-1	T304-4	11-8	Pex147-3	95-1	101-2	103-2	119-1	143-2	169-6	170-1	89-2	89-7	183-1	186-2	191-6	pCR106	Cm2
cap22388-1	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
qum18158-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+
pol17749-4	-	-	-	-	-	-	-	-	-	-	+[3]	-	-	+	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+
jam18349-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+
pnt17743-4	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	nd	+	-	-	-	+
pnt23011-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+
blb99-256-3	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+
blb2002	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+
blb8005-8	-	-	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
sto17605-4	-	-	-	-	-	-	-	+	+	+	+[3]	+[3]	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
pat18309-1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
sto17606-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	nd	-	-	-	-	+
hcb17719-1	-	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
ver17768-10	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
snk498042-1	-	-	-	-	-	-	-	-	nd	nd	nd	nd	-	+	+	+	-	-	-	+	+	+	+	+	+	nd	nd	-	+
AM3778-16	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+
moc2319-2	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+
avl18256-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	+	nd	-	-	-	-	+
cha962709-5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	nd	+	-	-	-	+
mcd23050-1	-	-	-	+[3]	+[3]	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	nd	-	-	-	-	+
mcd17596-8	-	-	-	+	-	+	-	-	+	+	+	-	-	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	+
mcd18200-6	-	-	-	+	-	-	-	-	-	+	+	-	-	+	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+
mcd21342-1	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	nd	-	-	-	-	+
oka18000-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	+	nd	+	-	-	-	+
Impala-*Rpi-blb1*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+
Impala	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
Désirée	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+
Bintje	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	-	nd	-	-	-	-	+

Table 1. Legend
A set of 56 RXLR effectors were inoculated by PVX agroinfection on 24 wild *Solanum* genotypes (corresponding to eight taxonomic series), three susceptible cultivars (Impala, Désirée and Bintje), and one resistant control, the transformant Impala-*Rpi-blb1*. Eight to sixteen replicates were inoculated, and necrotic responses occurring at frequencies higher (+) or lower (-) than 40% of the inoculated sites are presented. Candidate effectors are classified in specific vs. non-specific, representing candidates that showed response specifically in resistant plants or in both resistant and susceptible plants, respectively.
*Abbreviations for genebank source codes: CGN: Centre for genetic Resources, the Netherlands; PI: NRSP-6 - United States potato Genebank; GLKS: Gross Lusewitz, Germany.
[†] Resistant (R) or susceptible (S) to *P. infestans* isolate 90128.
[‡]Specific *Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1/1pi01&2* interactions
[a]nd, not determined
[§]Segregation of effector response in population, but not linked to resistance
[¶]Effector response detected in all progeny plants
[$]No effector response detected in progeny plants

Another tested combination, with mcd18200-6, resulted in the detection of the candidate effector in all progeny plants (see [¶] Table 1). This phenotype might be explained by homozygous presence of corresponding *R* gene(s) in the resistant parent. In five other combinations, the response to the candidate effector was not detected in progeny plants. These combinations represent perhaps 'false positives' due to over- expression of the effector by PVX in the high throughput screening of the parents (see [$] Table 1) (*Sol*Rgene database). In summary, we tested 13 PEXRD families of the 16 showing a response, and the number of effector candidates was reduced to three by genetic analysis: PEXRD11, PEXRD45, and PEXRD51.

We decided to focus on PEXRD11 (PITG_13930), because it caused cell death to snk498042-1, and to breeding clone AM3778-16 (Lokossou et al. 2009; Park et al. 2005c) the donor plant of *R2*-like gene that was reported to recognize PiAVR2 (Lokossou et al. 2009). PEXRD11 is represented by a functional clone 21-1 (51 amino acids (a.a.) after the EER motif), and by clone 43-1 that has an early stop codon in the putative functional domain (8 a.a. after the EER motif) (Oh et al. 2009). In the T30-4 reference genome of *P. infestans* (Haas et al. 2009), *PexRD11* is located in a gene-sparse, repeat-rich region that interrupts a region of colinearity between *P. infestans*, *P. sojae*, and *P. ramorum* (Figure 1A). This region is a typical genomic localization for RXLR-type and for other classes of effectors in *P. infestans* (Haas et al. 2009). PEXRD11 belongs to *P. infestans* RXLRfam32, which includes eight members, and is related to PiAVR2 (PITG_22870) of RXLRfam7, which includes an additional 18 members. Similar to *PiAvr2*, *PexRD11* is expressed at an early stage of infection in potato, i.e. at 16 hours post infection (Figure 1B), which is in line with previous studies (Haas et al. 2009; Oh et al. 2009; Whisson et al. 2007) and is consistent with features as an avirulence effector.

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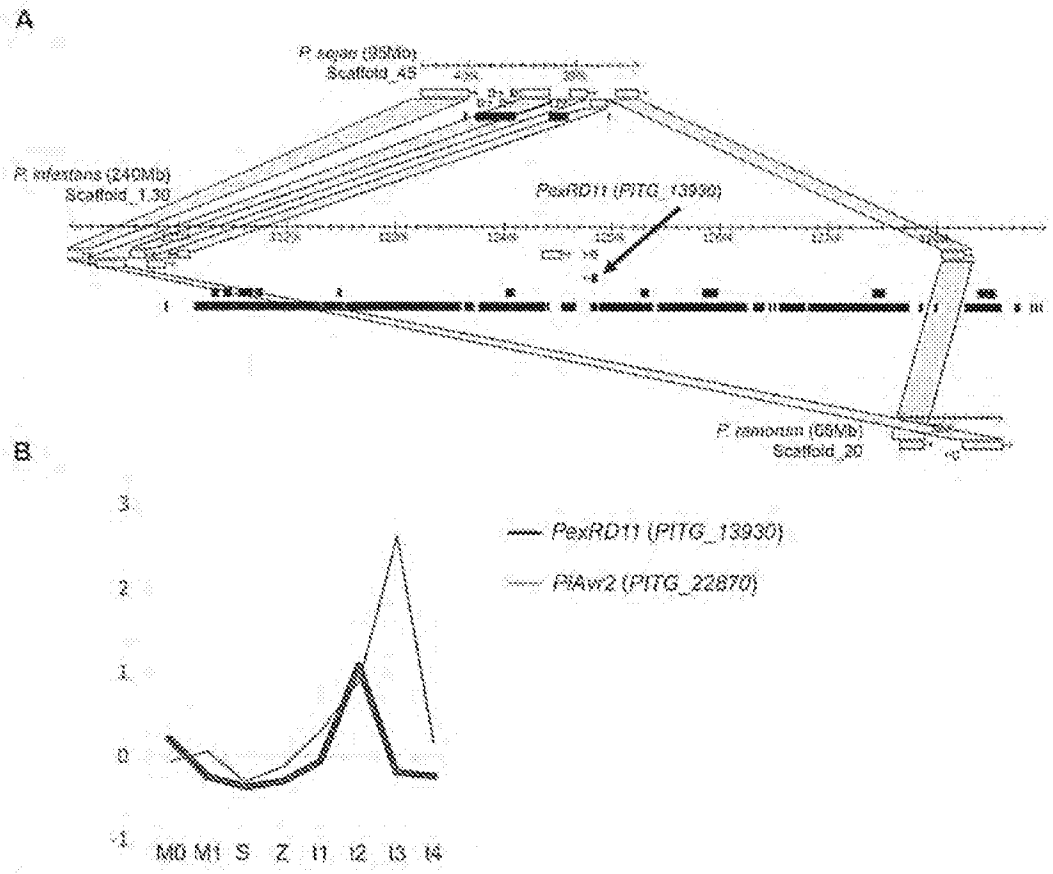


Figure 1. *PexRD11* has a similar pattern of expression and a similar protein structure compare to *PiAvr2*.

(A) *PexRD11* occurs in a gene-sparse, repeat-rich region flanked by regions of conserved colinearity between *Phytophthora sojae*, *P. infestans* and *P. ramorum*. Scaffold number from each genome is indicated. Genes are shown as turquoise boxes, *PexRD11* as red box, repeats as black boxes. Colinear orthologous gene pairs are connected by pink bands. (B) *PexRD11* is induced *in planta* at early stage of infection. Log2 normalization of *PexRD11* and *PiAvr2* expression profile from microarrays data is presented. Total RNA, of *P. infestans* T30-4 genome strain, isolated from mycelium grown, on Rye Sucrose Agar meduim (M0), on V8 medium (M1), from sporangia (S), zoospores (Z), infected leaves of potato, 6, 16 hours post inoculation, 2 and 5 days post inoculation (t1, t2, t3, and t4, respectively) were used. (C) Deduced amino acid sequences of PiAVR2 (PITG_08943 and PITG_22870) and PEXRD11 (PITG_13930) from *P. infestans* genome sequences database were aligned using the ClustalW software. Identical residues between PiAVR2 and PEXRD11 are shaded in dark grey, and conserved and semi-conserved residues are shaded in grey. Arrows point at RXLR and EER motif typical for cytoplasmic effectors. Underlined sequences are predicted signal peptide from SignalP 3.0 Server.

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Table 2. Functional allele-mining for response to PexRD11 in *Solanum*.

Series[a]	Origin	Solanum species	Gene bank [b]	Resistance [c]	Agroinfection			
					P. infestans 88128	21-1 pGR106:PEXRD11	23-1 pGR106:PEXRD11	neg. pGR106:empty	pos. pGR106:CRN2
Acaulia *S. demissum*	Bolivia	*S. acaule*	CGN 20820-4	9	-	-	-	-	+
	Argentina	*S. acaule*	CGN 17924-2	7	-	-	-	-	+
	Mexico	*S. acaule aemulans*	CGN 20568-4	7	-	-	-	-	+
	Peru	*S. albicans*	CGN 20867-3	5	-	-	-	-	+
	Mexico	*S. demissum*	CGN 17829-1	9	-	-	-	-	+
	Mexico	*S. demissum*	CGN 17828-2	9	-	-	-	-	+
	Mexico	*S. edinense*	PI 607474-3	9	+	-	-	-	+
Tuberosa Megistacroloba	Peru	*S. limoncochense*	GLKS 3828-2	1	-	-	-	-	+
	Unknown	*S. kurtzianum*	CPC 5889-3	9	-	-	-	-	+
	Bolivia	*S. megistacrolobum*	CGN 22347-1	7	-	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18145-4	9	-	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18146-5	9	-	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18147-2	9	-	-	-	-	+
	Peru	*S. mochiquense*	GLKS 2318-1	9	-	-	-	-	+
	Peru	*S. multidissectum*	CGN 17840-22	9	-	-	-	-	+
	Argentina	*S. species*	GLKS 018-23	3	-	-	-	-	+
Cultivated Tuberosa and Tuberosa from Peru	Bolivia	*S. candolleanum*	CGN 18132-3	3	-	-	-	-	+
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. acroc-pepo*	GLKS 82-1	7	-	-	-	-	+
	Bolivia	*S. astleyi*	CGN 18211-4	3	-	-	-	-	+
	Bolivia	*S. avilesii*	CGN 18258-2	6	-	-	-	-	+
	Bolivia	*S. berthaultii*	CGN 18190-3	9	-	-	-	-	+
	Bolivia	*S. chacoense*	CGN 18385-2	9	-	-	-	-	+
	Bolivia	*S. doddsii*	PI 545854-5	6	-	-	-	-	+
	Bolivia	*S. doddsii*	PI 545854-3	6	-	-	-	-	+
	Bolivia	*S. gourlayi pachypricbum*	CGN 18188-2	1	-	-	-	-	+
	Argentina	*S. gourlayi vidaurrei*	CGN 23045-2	9	-	-	-	-	+
	Argentina	*S. hawkesianum*	CGN 17888-4	7	-	-	-	-	+
	Argentina	*S. incamayoense*	CGN 21329-1	7	-	-	-	-	+
	Argentina	*S. incamayoense*	CGN 17875-5	7	-	-	-	-	+
	Argentina	*S. incamayoense*	CGN 17968-4	6	-	-	-	-	+
	Bolivia	*S. iopbyes*	CGN 18173-5	7	-	-	-	-	+
	Argentina	*S. microdontum*	CGN 17596-8	7	-	-	-	-	+
	Argentina	*S. microdontum*	CGN 17596-1	3	-	-	-	-	+
	Argentina	*S. microdontum*	CGN 21342-1	7	-	-	-	-	+
	Argentina	*S. neocasii*	CGN 18280-1	2	-	-	-	-	+
	Peru	*S. raphanifolium*	CGN 18088-5	8	-	-	-	-	+
	Peru	*S. ruiz-lealii*	CGN 18117-1	4	-	-	-	-	+
	Peru	*S. species*	CGN 20580-1	5	-	-	-	-	+
	Bolivia	*S. sucrense*	CGN 18026-1	6	-	-	-	-	+
	Bolivia	*S. sucrense*	CGN 18026-2	5	-	-	-	-	+
	Bolivia	*S. tarijense*	CGN 22729-5	8	-	-	-	-	+
	Argentina	*S. vernei*	CGN 18112-6	9	-	-	-	-	+
	Bolivia	*S. virgultorum*	CGN 17775-3	8	-	-	-	-	+
S. verrucosum	Mexico	*S. brachycarpum*	CGN 18347-8	9	-	-	-	-	+
	Mexico	*S. verrucosum*	CGN 22374-2	9	-	-	-	-	+
Demissa Conicibaccata	Mexico	*S. agrimoniifolium*	PI 545748-1	9	-	-	-	-	+
	Mexico	*S. brachycarpum*	CGN 17721-2	8	-	-	-	-	+
	Mexico	*S. brachycarpum*	CGN 17721-3	8	-	-	-	-	+
	Colombia	*S. colombianum*	CGN 18289-3	8	-	-	-	-	+
	Mexico	*S. hougasii*	CPC 7030-1	9	-	-	-	-	+
	Mexico	*S. hougasii*	CGN 18339-2	8	-	-	-	-	+
	Unknown	*S. lopezianum*	CPC 2922-1	9	-	-	-	-	+
	Costa Rica	*S. longiconicum*	PI 310994-3	7	-	-	-	-	+
	Mexico	*S. schenckii*	PI 498042-1	8	+	-	-	-	+
	Venezuela	*S. subpandurarum*	PI 498289-3	4	-	-	-	-	+
	Ecuador	*S. tandilianense*	PI 473478	3	-	-	-	-	+

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Table 2. (*Continued*) **Functional allele-mining for response to PexRD11 in *Solanum*.**

Series[a]	Origin	Solanum species	Gene bank *	Resistance [b]	Agroinfection			
					P. infestans 90128	pGR106::PEXRD11 21-1	pGR106::PEXRD11 43-1	neg. pGR106::empty	pos. pGR106::CRN2
Longipedicellata	Argentina	*S. gourlayi*	CGN 22380-1	9	-	-	-	+
	Mexico	*S. hjertingii*	CGN 17717-3	8	+	+	-	+
	Mexico	*S. papita*	CGN 17830-1	9	-	-	-	+
	Mexico	*S. papita*	CGN 17832-2	9	-	-	-	+
	Mexico	*S. papita*	CGN 17831-1	9	-	-	-	+
	Mexico	*S. papita*	CGN 17831-8	9	-	-	-	+
	Mexico	*S. papita*	CGN 18319-1	4	-	+	-	+
	Mexico	*S. papita*	CGN 18319-4	6	-	-	-	+
	Mexico	*S. polytrichon*	CGN 17750-1	6	-	+	-	+
	Mexico	*S. polytrichon*	CGN 17750-2	7	-	-	-	+
	Mexico	*S. polytrichon*	CGN 17750-8	7	-	-	-	+
	Chile	*S. species*	CPC 7211-4	9	-	-	-	+
	Unknown	*S. species*	CPC 7328-2	9	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 17805-4	7	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18332-1	9	-	-	-	+
	Peru	*S. stoloniferum*	CGN 22718-2	9	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 23581-3	8	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18348-6	7	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18333-6	7	-	-	-	+
Circaeifolia Piurana Megistacroloba Tuberosa	Ecuador	*S. albicans*	PI 561633-2	6	-	-	-	+
	Bolivia	*S. capsicibaccatum*	CGN 22388-1	9	-	-	-	+
	Peru	*S. chomatophilum*	CGN 18356-2	8	-	-	-	+
	Bolivia	*S. circaeifolium*	CGN 18133-3	2	-	-	-	+
	Bolivia	*S. circaeifolium quimense*	CGN 18137-6	3	-	-	-	+
	Peru	*S. dolichocremastrum*	PI 498236-1	9	-	-	-	+
	Peru	*S. huancabambense*	CGN 17719-1	9	-	-	-	+
	Peru	*S. huancabambense*	CGN 17719-2	1	-	-	-	+
	Peru	*S. immite*	PI 365330-4	9	-	-	-	+
	Peru	*S. mochiquense*	CGN 18283-3	7	-	-	-	+
	Peru	*S. piurana*	PI 473501-1	9	-	-	-	+
Pulyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. bulbocastanum*	CGN 21308-10	8	-	-	-	+
	Mexico	*S. bulbocastanum*	CGN 17693-2	9	-	-	-	+
	Guatemala	*S. bulbocastanum*	CGN 23075-1	9	-	-	-	+
	Guatemala	*S. bulbocastanum pertitum*	PI 275200-2	4	-	-	-	+
	Mexico	*S. cardiophyllum*	CGN 22387-2	9	-	-	-	+
	United States	*S. jamesii*	CGN 18349-1	9	-	-	-	+
	United States	*S. jamesii*	CGN 18349-10	9	-	-	-	+
	Mexico	*S. michoacanum*	CLKS 2346-2	8	-	-	-	+
	Bolivia	*S. neocardenasii*	CGN 18217-2	9	-	-	-	+
	Mexico	*S. pinnatisectum*	CGN 23011-1	8	-	-	-	+
	Mexico	*S. polyadenium*	CGN 17749-4	7	-	-	-	+
	Mexico	*S. tarnii*	PI 545742-3	7	-	-	-	+
Out group	Bolivia	*S. chiquidenum*	CGN 18060-1	9	-	-	-	+
	Costa Rica	*S. nigrum*	CGN 21307-1	9	-	-	-	+

100 *Solanum* genotypes belonging to various phylogenetic series (Jacobs et al. 2008) and from different countries were tested by PVX agroinfection for response to pGR106::PEXRD11 clone 21-1, pGR106::PEXRD11 clone 43-1, pGR106::empty vector and pGR106::CRN2. At least 16 replicates were inoculated, and necrotic responses occurring at frequencies higher (+) or lower (-) than 40% of the inoculated sites are presented.

n.d. not determined

[a] Series according to Jacobs (2008)

*Abbreviations for genebank source codes: see Table 1.

[b] Resistance evaluated in field and/or detached leaf assay (*Sol*Rgene database)

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Functional allele mining reveals specific response to PEXRD11 in *S. edinense*, and *S. hjertingii*.

To identify additional *Solanum* species responding to PEXRD11, we selected a set of 144 genotypes: 142 genotypes of *Solanum* species section *Petota* and two genotypes outside the *Petota* as controls: *S. chaparense* and *S. nigrum*. These 144 plants represents genotypes from North, Central, and South America, covering all taxonomic series, as described by Jacobs et al. (2008), and covering different degrees of resistance to *P. infestans* isolate 90128 (Table 2), as obtained in a high throughput screen of germplasm (*SolR*gene database). From each genotype, we performed a PVX agroinfection with PEXRD11 clones 21-1 and 43-1, the negative control pGR106::empty, and the positive control pGR106::CRN2. Forty-four genotypes showed either response to pGR106::empty, or no response to pGR106::CRN2, and were removed from the analyses. Within the remaining 100 *Solanum* genotypes, we identified a specific response to pGR106::PEXRD11 clone 21-1 in *S. edinense* (edn) 607474-3 and *S. hjertingii* (hjt) 17717-3, in addition to snk498042-1 control (Table 2 and Figure 2). The identified species belong to one of the three phylogenetic series: Acaulia, Demissa/Conicibaccata, and Longipedicellata (Jacobs et al. 2008), which suggests that recognition of PEXRD11 is dispersed over polyploid *Petota* species (Table 2).

Response to PEXRD11 in edn607474-3 maps to the *R2* cluster.

In addition to edn607474-3, hjt17717-3, and snk498042-1, the breeding clone AM3778-16 also responds to PEXRD11 by PVX agroinfection (Table 1). The *R2*-like gene from AM3778-16 was located previously on an MLB locus on LG IV (Lokossou et al. 2009; Park et al. 2005b; Park et al. 2005c), where *R2*, *Rpi-abpt* and *Rpi-blb3* also reside. To investigate whether this locus also contains a functional *Rpi* gene in edn607474-3, we generated a population of 190 F1 genotypes and inoculated them with *P. infestans* isolate 90128. A 1:1 segregation for resistance was evident and pointed to presence of a dominant *R* gene, designated *Rpi-edn1*. We tested eight resistant and eight susceptible F1 genotypes and their parents for response to pGR106::PEXRD11, and found a 100% correlation between resistance to *P. infestans* isolate 90128 and response to PEXRD11 (21-1) (Table 3). This correlation leads to the hypothesis that *Rpi-edn1* might be responsible for the response to PEXRD11 and might be located in the *R2* cluster of LG IV. To test this hypothesis, a set of PCR markers mapping on the short arm of LG IV were examined on the population. SSR marker RGH-SSR51 showed linkage with resistance to *P. infestans*, with 26 recombinants out of 142 F1 individuals (18cM), and was present in the eight resistant genotypes responding to PEXRD11 (21-1), but not in the eight susceptible, non-responding plants (Table 3). This finding indicates that *Rpi-edn1* is located on LG IV, possibly in the *R2* cluster. To identify more closely linked markers, we designed primers corresponding to the NBS region of *R2*, and we identified one marker (R2-2350-Rsa) linked with resistance to *P. infestans* with 2 recombinants out of 93 F1 individuals (2cM). The Th21 marker, which is located in the *R2*

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Table 3. Co-segregation for resistance, PexRD11 response and *R2* genetic markers in the edn607474-3 population.

Genotype	A	B				C		
	Resistance test	PVX Agroinfection				Markers		
		PEXRD11		negative	positive	HRM	SSR	NBS
	P. infestans inoculation 90128	pGR108 ::21-1	pGR106 ::43-1	pGR106 ::empty	pGR106 ::CRN2	Th21	RGH-SSR51	R2-2350-Rsa
edn607474-3	R	+	-	-	+	+	+	+
Concurrent	S	-	-	-	+	-	-	-
Offspring								
104	S	-	-	-	+	-	-	n.d.
108	S	-	-	-	+	-	-	-
111	R	+	-	-	+	+	+	+
112	R	+	-	-	+	+	+	+
114	R	+	-	-	+	+	+	+
115	R	+	-	-	+	+	+	+
116	S	-	-	-	+	-	-	-
117	S	-	-	-	+	n.d.	-	-
119	S	-	-	-	+	-	-	-
120	R	+	-	-	+	+	+	+
122	R	+	-	-	+	+	+	+
123	R	+	-	-	+	+	+	+
124	R	+	-	-	+	+	+	+
125	S	-	-	-	+	-	-	-
126	S	-	-	-	+	-	-	-
128	S	-	-	-	+	-	-	-

(A) Resistance (R) or susceptibility (S) against *P. infestans* isolate 90128. (B) Presence (+) or absence (-) of cell death around the inoculation site with *A. tumefaciens* clones expressing pGR106::PEXRD11 clones 21-1 and 43-1, pGR106::CRN2 (positive control) and the pGR106::empty vector (negative control) at 14 days post inoculation. (C) Presence (+) or absence (-) of the polymorphic band of the genetic HRM marker TH21 (Park et al. 2005b), the SSR marker RGH-SSR51 and profiling marker R2-2350-Rsa. n.d. not determined.

cluster (Park et al. 2005b), also showed linkage with resistance to *P. infestans*, with 6 recombinants out of F1 126 individuals (5cM). R2-2350-Rsa and Th21 fully co-segregated with resistance and effector recognition in the eight resistant genotypes tested with PEXRD11 (Table 3), suggesting that *Rpi-edn1* is located in the *R2* cluster and may be an *R2* homologue.

Snk498042-1 has been shown to contain *Rpi-snk1* on LG IV and an *R* gene in a population that was derived from Snk498042-1 and *S. brachycarpum* (CGN: 18347-8) (Jacobs et al. 2010). *Rpi-snk1* was linked to the CAPS marker Th21, and, was present in 2/3 of resistant offspring (Jacobs et al. 2010). Because bcp18347-8 was resistant to PVX, we used another population, derived from a cross with *S. stoloniferum* (sto0012-1), to investigate segregation of response to PEXRD11 effector. We found again 2/3 of resistant offspring responding to PEXRD11 (21-1), which led us to postulate that *Rpi-snk1* is also potentially an *R2* homologue that responds to PEXRD11.

Crosses with resistant parent hjt17717-3 were not successful, and therefore, genetic mapping of *Rpi-hjt1* was not further pursued. Nevertheless, we showed that edn607474-3 contained *Rpi-edn1* and snk498042-1 contained *Rpi-snk1*, and that both are located on the short arm of LG IV, in the same locus as *R2-like* (AM3778-16). Both *R* genes are potentially

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homologues of *R2* and respond to pGR106::PEXRD11. We postulate, therefore, that hjt17717-3 might also contain an *R2* homologue, because it also responds to PEXRD11.

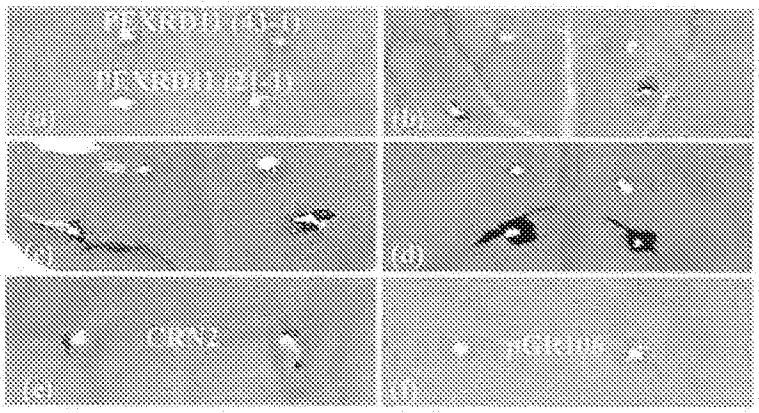


Figure 2. PEXRD11 induces cell death in *S. edinense* 607474-3, *S. schenckii* 498042-1 and *S. hjertingii* 17717-3.
PVX agroinfection was conducted on leaves of *S. tuberosum* cv. *Concurrent* (a), edn607474-3 (b), snk498042-1 (c) and hjt17717-3 (d) by wound-inoculation with *A. tumefaciens* strains carrying pGR106::PEXRD11 (21-1), and pGR106::PEXRD11 (43-1). Positive and negative controls, pGR106::CRN2 and pGR106::empty respectively, are shown on potato cultivar Concurrent (e and f). Pictures were taken at 12 and 16 days post inoculation.

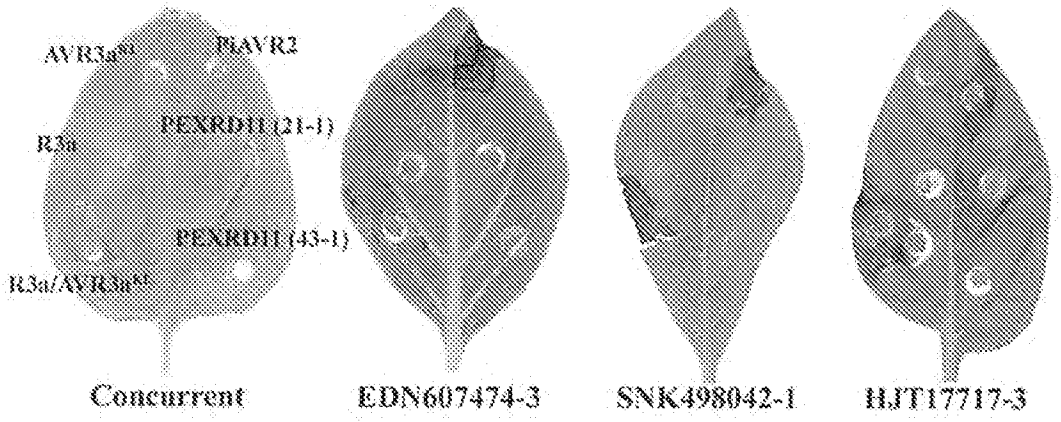


Figure 3. PiAVR2 induces cell death in *S. edinense* 607474-3, *S. schenckii* 498042-1 and *S. hjertingii* 17717-3.
Agroinfiltration using *A. tumefaciens* strain carrying, *PiAvr2*, *PexRD11* (21-1) and *PexRD11* (43-1) independently, was performed in *Solanum tuberosum cv. Concurrent*, edn607474-3, snk498042-1 and hjt17717-3. *R3a/Avr3a*^{KI} co-infiltration, and *R3a* and *Avr3a*^{KI} single infiltrations were included as positive and negative controls, respectively. Photographs of symptoms were taken at four dpi.

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Hjt17717-3, edn607474-3 and snk498042-1 respond to PiAVR2.

The *R2*, *R2-like*, *Rpi-abpt*, and *Rpi-blb3* genes from the *R2* cluster specifically induce cell death when coinfiltrated with PiAVR2 from *P. infestans* (Lokossou et al. 2009). PEXRD11 and PiAVR2 proteins showed a high level of protein-sequence similarity, with 63% of similarity based on identical, conserved and semi-conserved amino acid substitutions (Figure 1C). To test the presence of a functional *R2* homologue in hjt17717-3, edn607474-3, and snk498042-1, we infiltrated *A. tumefaciens* strains expressing PiAVR2 in *Solanum* leaves. In the three wild *Solanum* genotypes, agroinfiltration with *PiAvr2* resulted in a specific cell death response at four days post infiltration (Figure 3).

In the same experiment, we included PEXRD11 clone 21-1 and 43-1 to test whether the results obtained with the PVX agroinfection could be confirmed by agroinfiltration (Figure 2 and 3). In hjt17717-3, PVX agroinfiltration of PEXRD11 (21-1) resulted in cell death. In edn607474-3 and snk498042-1, however, agroinfiltration with PEXRD11 (21-1) was not sensitive enough to result in cell death (Figure 3). In summary, the agroinfiltration experiments showed that specific cell death was always induced with *PiAvr2*, which indicates presence of functional *R2* homologues in hjt17717-3, edn607474-3, and snk498042-1, and might suggest some degree of functional redundancy between PiAVR2 and PEXRD11.

Allele mining in edn607474-3, snk498042-1 and hjt17717-3 reveals new variants of the *R2* family.

To identify which *R* genes in edn607474-3, snk498042-1, and hjt17717-3 were responsible for response to PiAVR2 or PEXRD11 (21-1) or both, we followed an allele-mining approach. PCR with conserved *R2* primers on genomic DNA of the three wild *Solanum* plants yielded amplicons of 2500bp, which is in the range of the expected size of an *R2* homologue. From hjt17717-3, additional amplicons of 3200bp were obtained that seemed to contain a hypothetical intron of about 750bp in the NBS region (data not shown). To determine the presence or absence of the intron, as well as the expression of these potential genes, new PCR amplifications were made on cDNA for this genotype, and amplicons were cloned and sequenced; each sequence was about 2500bp. Sequence analysis of the amplicons of the three *Solanum* species revealed eight unique *RGH* sequences from edn607474-3 (*ednR2GH*), seven from snk498042-1 (*snkR2GH*), and six from hjt17717-3 (*hjtR2GH*), with amino acid identities from 82.4% to 100% with *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* (Table 4). Nucleotide sequences identical to *R2*-like were retrieved from edn607474-3 (*ednR2GH2*) and from hjt17717-3 (*hjtR2GH6*). *hjtR2GH1*, furthermore, is identical to *snkR2GH1* and *hjtR2GH4* is identical to *ednR2GH6*, which illustrates a degree of redundancy and origin of the *R2* homologues in *Solanum*.

The phylogenetic relationship between the R2 variants was examined by constructing a neighborjoining (NJ) tree (Figure 4). The NJ tree rooted with R3a, included RPP13 sequence from *Arabidopsis thaliana*, the closest known homologue of R2 in plants (Bittner-

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Eddy et al. 2000; Lokossou et al. 2009). The R2 variants were grouped in six clades: clade I to clade VI. Clade I contains the four known functional R2 homologues, R2, Rpi-blb3, Rpi-abpt, and R2-like (ednR2GH2, hjtR2GH6), plus three new variants, ednR2GH1, hjtR2GH3, and snkR2GH3. Clade II contains hjtR2GH2, -GH5, and snkR2GH4; clade III contains the two identical snkR2GH1 and hjtR2GH1, as well as snkR2GH2; and clade IV contains snkR2GH5, -GH6, -GH7, and ednR2GH4. EdnR2GH-3 from edn607474-3 is different from any other variant, and represents clade V. Clade VI is the most distant from the original R2, and it contains ednR2GH5, -GH6, -GH7, -GH8, and hjtR2GH4. In summary, the three wild *Solanum* species contain an extended and diverse number of R2 variants, some of which are close to the known R2 family genes. In addition, five new clades representing 14 additional variants were identified.

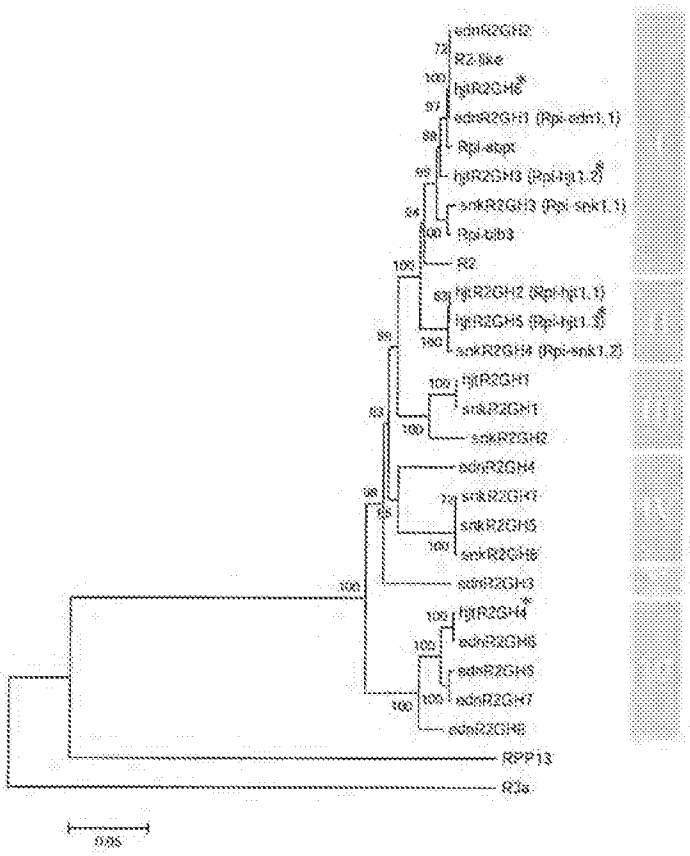


Figure 4. The *R2* family is composed of a high number of diverse of variants.
The neighbor-joining tree is illustrating phylogenetic relationship at the amino acid level between R2 variants from edn607474-3, snk498042-1 and hjt17717-3 with R2, R2-like, Rpi-abpt Rpi-blb3 and RPP13 vs. R3a as out group. Sequences are grouped in six clades. Sequences with a * were amplified from cDNA. Bootstrap values greater than 60% (based on 1000 resample) are indicated at the nodes.

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Table 4. Percentage similarity between all RGH sequences used in the phylogenetic tree in Figure 4.

	ednR2GH1	ednR2GH2	ednR2GH3	ednR2GH4	ednR2GH5	ednR2GH6	ednR2GH7	ednR2GH8	hjtR2GH1	hjtR2GH2	hjtR2GH3	hjtR2GH4	hjtR2GH5	hjtR2GH6	snkR2GH1	snkR2GH2	snkR2GH3	snkR2GH4	snkR2GH5	snkR2GH6	snkR2GH7	Rpi-blb3	R2	R2-like	Rpi-abpt	RPP13	R3a
ednR2GH1	***	99.9	85.1	85.6	83.1	82.9	83.2	83.2	87.7	94.6	98.7	82.9	84.7	99.9	87.7	87.2	96.1	84.4	86.9	86.7	85.6	97.2	94.6	99.9	99.8	38	27.3
ednR2GH2	0.1	***	85	85.5	83	82.8	83.1	83.1	87.9	94.7	98.8	82.8	84.6	100	87.9	87.3	96.2	84.5	87	86.9	85.7	97.3	94.4	100	99.9	38	27.1
ednR2GH3	15.7	15.8	***	84.7	80.6	80.2	80.7	85.7	84.5	85.3	85.2	80.2	85.4	85	84.5	84.3	85.7	85.3	86.1	86	85.9	86.2	84.1	85	84.5	35.4	26.9
ednR2GH4	16	15.8	17.2	***	79.8	79.8	80	80.8	84.2	86.2	85.6	79.8	86.4	85.8	84.2	84.4	84.3	86.1	88.2	88.1	88	84.8	87.9	85.8	85.1	36.8	27
ednR2GH5	19.2	19.3	22.5	23.6	***	97.1	89.6	92.8	81.1	83	82.9	97.1	83.1	83	81.1	80.3	82.4	83.1	81	81	80.7	82.5	83.4	83	83.5	36.7	27
ednR2GH6	19.5	19.6	23	23.6	2.9	***	97.5	94.5	81	82.9	82.6	100	83	82.8	81	80.2	82.3	82.9	81.5	81.5	81.3	82.4	83	82.6	83.4	36.3	27.4
ednR2GH7	19	19.2	22.4	23.4	0.3	2.5	***	92.9	81.2	83.1	83	97.5	83.2	83.1	81.2	80.3	82.5	83.2	81.1	81.1	80.8	82.6	83.5	83.1	83.7	36.5	27
ednR2GH8	19	19.2	15.9	23.3	7.6	5.7	7.6	***	81.2	82.8	83	94.5	82.8	83.1	81.2	80.8	83.8	82.6	82.4	82.4	82.7	84.2	83.5	83.1	83.7	36.7	27.2
hjtR2GH1	13.4	13.3	17.4	17.8	21.8	22	21.7	21.7	***	87.5	87.7	81	87.6	87.9	100	91.6	86.8	87.1	85.3	85	84.9	87	85.7	81.9	87.1	36.2	26.3
hjtR2GH2	5.6	5.5	16.2	15.2	19.3	18.5	19.2	18.8	13.7	***	98.8	82.9	99.9	94.7	87.5	87.2	93.8	98.4	86.3	86.2	86	94.1	92.7	94.7	93.5	36.2	27.5
hjtR2GH3	1.3	1.2	16.5	16	19.5	19.6	19.3	19.3	13.4	4.3	***	82.6	98	98.8	87.7	87.4	95.6	98.5	87.7	87	86.9	96.6	93.3	98.6	97.6	36.8	27
hjtR2GH4	19.5	19.6	23	23.5	2.9	0	2.9	5.7	22	19.5	19.8	***	83	82.8	81	80.2	82.3	82.9	81.5	81.3	82.4	83	82.6	83.4	36.3	27.4
hjtR2GH5	5.5	5.4	16.2	15.1	19.2	18.3	19	19.5	13.6	0.1	4.1	19.3	***	94.8	87.6	87.3	93.9	99.3	86.3	86.2	86	94.2	92.8	94.8	93.7	36.2	27.5
hjtR2GH6	0.1	0	16.8	15.8	19.3	19.6	19.3	19.2	13.3	5.5	1.2	19.6	5.4	***	87.9	87.3	96.2	94.5	87	86.9	86.7	97.3	94.4	100	98.9	36	27.1
snkR2GH1	13.4	13.3	17.4	17.8	21.8	22	21.7	21.7	0	13.7	13.4	22	13.6	13.3	***	91.6	86.6	87.1	85.1	85	84.9	87	85.7	87.9	87.1	36.2	26.3
snkR2GH2	14.1	13.9	17.7	17.6	23.1	23.1	22.9	23.3	8.8	14.1	13.8	23.1	13.9	13.9	8.8	***	85.8	87	85.5	85.5	85.4	86.3	86.2	87.3	86.6	36.4	27
snkR2GH3	4	3.9	15.9	17.6	20.1	20.3	20	18.3	14.9	6.5	4.4	20.3	6.4	3.9	14.9	15.8	***	93.5	85.6	85.4	85.3	86.6	92.1	96.2	95.1	36	27.4
snkR2GH4	5.8	5.7	16.4	15.5	19.3	19.4	19.1	19.9	14.2	0.8	4.6	19.4	6.7	5.7	14.2	14.4	6.7	***	86.5	86.3	86.2	94	92.5	94.6	93.5	36.1	27.5
snkR2GH5	14.5	14.3	15.4	12.8	22	21.2	21.9	20.1	16.7	15.2	14.2	21.2	15.2	14.3	16.7	16	15.1	15	***	99.9	99.8	86	84.9	87	86.1	35.2	26.4
snkR2GH6	14.6	14.5	15.5	13	22	21.2	21.9	20.1	16.8	15.3	14.3	21.2	15.3	14.5	16.8	16.2	15.2	15.7	0.1	***	99.6	86.2	84.8	86.9	86	35.2	26.4
snkR2GH7	14.8	14.6	15.7	13.1	22.4	21.6	22.2	20.5	17	15.5	14.5	21.6	15.5	14.6	17	16.3	15.4	15.3	0.2	0.3	***	85.8	84.7	86.7	85.9	35	26.1
Rpi-blb3	2.8	2.8	15.3	16.8	20	20.1	19.8	17.8	14.3	6.2	3.5	20.1	6	2.8	14.3	15.2	1.3	6.3	15.5	15.6	15.8	***	92.9	97.3	96.2	36.3	27.1
R2	5.7	5.6	17.9	13.3	18.8	18.3	18.7	18.7	15.9	7.7	7.1	19.3	7.6	5.8	15.9	15.3	5.4	7.9	16.9	17	17.2	7.5	***	94.4	95.5	36.6	27.3
R2-like	0.1	0	16.8	15.9	19.3	19.6	19.2	19.2	13.3	5.5	1.2	19.6	5.4	0	13.3	13.9	3.8	6.7	14.3	14.5	14.8	2.8	5.6	***	98.9	36	27.1
Rpi-abpt	1.2	1.1	17.5	16.8	18.5	18.9	18.6	18.4	14.2	6.7	2.3	18.9	6.8	1.1	14.2	14.5	5	6.8	15.5	15.6	15.7	3.9	4.6	1.1	***	36.1	27
RPP13	127.8	127.8	130.6	124.5	124.8	126.5	125.4	128.2	127.1	127.2	128.8	126.5	127.2	127.8	127.1	126.3	127.8	127.7	131.8	131.5	132.8	126.7	125.2	127.8	127.4	***	28.6
R3a	179.2	180.1	182.2	181	181.4	177.7	181.4	179.5	187.3	177.3	181.1	177.7	177.3	180.1	187.2	181	178.3	179.5	185.4	186.4	188.4	180.1	178.7	180.1	181.5	217	***

Amino acid sequence distances of the 21 R2 variants of edn607474-3, snk498042-1 and hjt17717-3 with R2, R2-like, Rpi-abpt, Rpi-blb3 compared to RPP13 and R3a. The percentage of similarity is in the upper triangle, the percentage of divergence is in the lower triangle.

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***R2* is under diversifying selection.**

Alignment of amino acids sequences of the 17 new *R2* variants with *R2*, *R2-like*, *Rpi-abpt*, and *Rpi-blb3* revealed two groups of sequence affiliation (Figure 5). All R2 variants in clade I and II had a mostly conserved LRR domain; only LRR repeats 3, 6, and 14 displayed one single nucleotide polymorphism (SNP) each (Figure 5). In contrast, the 11 other variants contained multiple SNPs throughout the entire LRR domain. To investigate which region of R2 was under diversifying selection, we applied the PAML method (Yang et al. 2005) and identified several positively selected amino acid residues (posterior probability, P>95%), of which the majority were in the LRR domain (Figure 6). Model M2 identified 19 amino acid residues under positive selection (Table 5), 15 of which were present in the LRR domain. The selection model M8 gave the same log likelihood value as M2 and identified the same 19 positively selected residues plus 22 additional a.a. of which 12 were in the LRR domain (Figure 6), for a total of 27 in the LRR domain. Most positively selected amino acid residues with a high posterior probability (P>99%) in the LRR domain were in LRR repeats 8, 10, 11, 12, and 13; on the border of LRR repeats 5 and 9; and in LRR subdomains 4/5, 5/6, and 6/7. In addition to the 27 sites in the LRR domain, six sites in the Leucine Zipper and eight in the NB-ARC domain, with one amino acid in the Kinase 2, were also under positive selection.

Table 5: Positive selection analysis among R2 variants.

Model of selection	Estimated parameters	l[a]	Model comparison	2Dl (df,p)[b]	Positively diversified codons[c]
M0: one ratio	$w = 0.637$	-9429.41			Not allowed
M1: neutral	$r_0 = 0.619$; $r_1 = 0.381$ $w_0 = 0.034$; $w_1 = 1$ (fixed)	-9194.64			Not allowed
M2: positive selection	$r_0 = 0.582$; $r_1 = 0.320$; $r_2 = 0.097$ $w_0 = 0.040$; $w_1 = 1.000$; $w_2 = 4.015$	-9124.45	M1 vs. M2	140.4 (2, <0.001)	**32R 134N 254G** 267R 598H **620T 621F** 622F 627C 648C **681S 683D** 684R 686R 710R 751S **752F** 776G 799R
M7: beta	$r = 0.018$; $q = 0.025$	-9196.00			Not allowed
M8: beta & w	$r_0 = 0.897$; $r = 0.080$; $q = 0.138$ $r_1 = 0.103$; $w = 3.931$	-9124.53	M7 vs. M8	142.9 (2, <0.001)	25K 29D **32R** 107A **134N** 136A 145T 204T 209L 210N 240R **254G 267R** 341A 550R 553S 557L **598H 620T 621F** 622F 624E **627C** 643T **648C** 662A **681S 683D 684R 686R 710R** 715S 731Q **751S 752F** 754E 773K 775D **776G 799R** 802W

[a] Log likelihood value

[b] Likelihood ratio test: $2\Delta l = 2(\ln l_{\text{alternative hypothesis}} - \ln l_{\text{null hypothesis}})$, with significance evaluated from χ^2 distribution; df is degree of freedom and p is the probability

[c] Bayes Empirical Bayes (BEB) analysis (Yang et al. 2005); Amino acid sites, based on *R2-like* sequence, inferred to be under diversifying selection with probability >95% and >99% in bold

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Figure 3. Protein alignment of R2 variants reveals a mainly conserved LRR domain in clade I/II R2 variants.

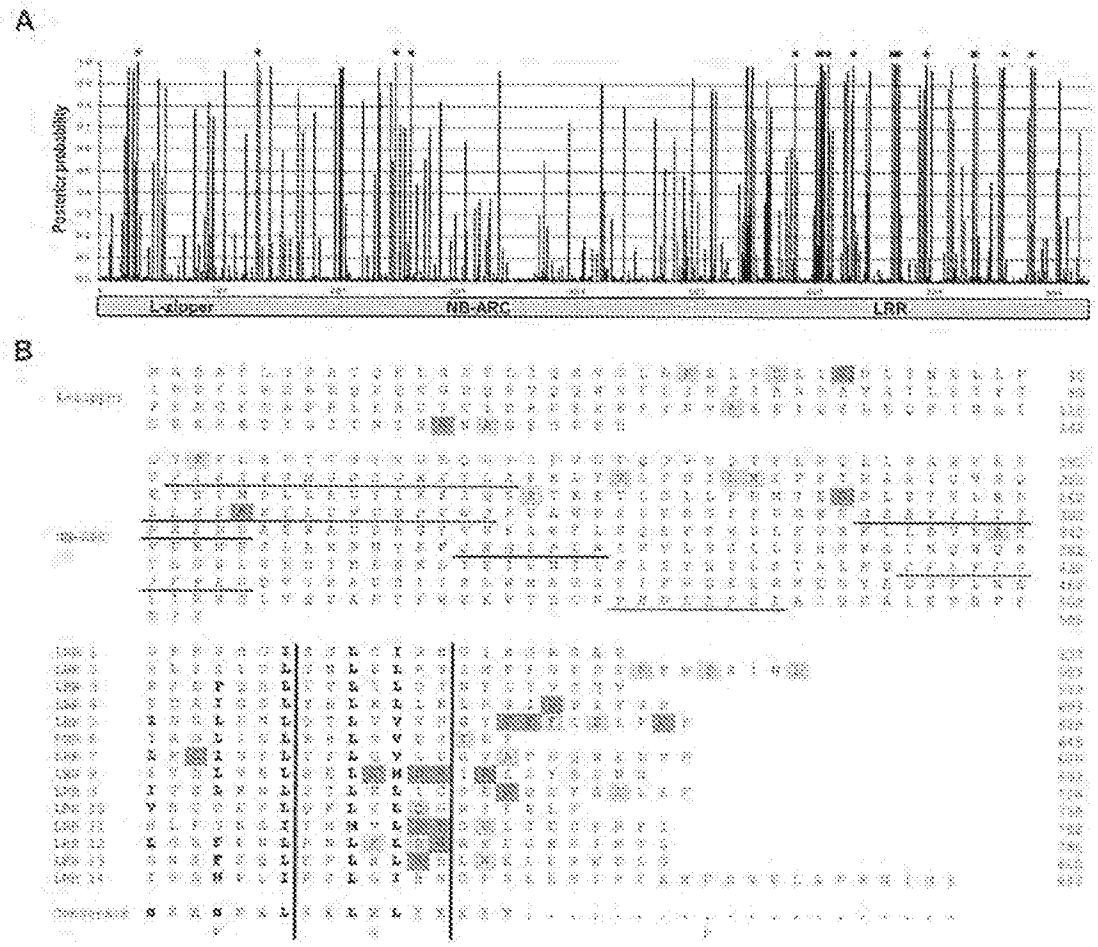


Figure 6. Positive selection in *R2* variants has mostly targeted the LRR domain.
(A) Graphical representation of posterior probability of diversifying selection based on model M8 at each site in R2 variants. The * indicates a posterior probability greater than 99% of having $\omega > 1$. The *x* axis denotes codon position in the alignment of R2 variants made from codeml and removing all gaps. (B) Amino acids with posterior probability of diversifying selection greater than 95% (highlight in clear-grey) or greater than 99% (highlight in dark-grey) are presented on the sequence of R2-like. In the NB-ARC domain, characteristic conserved domains i.e. kinase 1, kinase 2, kinase 3, GLPL, RNBS-D and VHD motifs in that order, are underlined and in italic. The leucine-rich repeat domain is composed of 14 repeats, with the putative β-strand/β-turn motif, xxLxxLxx, in which the x residues are solvent-exposed residues available for interactions with potential ligands (Zhou et al. 2006). In the consensus, L. represents any one of five hydrophobic amino acids (F, I, L, M or V), X represents any amino acid, α. indicates sites with mostly hydrophobic amino acids (F, I, L, M, V, Y, T, H or C), a. designate the LRR repeats motif enclosed between the two vertical lines, and b. point the subdomains which may form a potential connecting βα loop (Jones et al. 1997).

Figure 5. (*Previous page*) **Protein alignment of R2 variants reveals a mainly conserved LRR domain in clade I/II R2 variants.**
The R2-like amino acid sequence is used as reference and only positional a.a. changes are noted for the other sequences. The leucine zipper domain is in italics with the first and the fifth hydrophobic residues from the heptads in bold and underlined, respectively (Lokossou et al. 2009). The NB-ARC domain includes the P-loop (kinase 1), kinase 2, and kinase 3 motifs of the NBS and GLPL, RNBS-D and MHDV motifs underlined that are conserved in NBS-LRR proteins (Bendahmane et al. 2002; Meyers et al. 1999; van der Biezen and Jones 1998). The 14 LRR repeats are presented in a grey box. A black line separates sequences in clade I and II from those in clade III to VI.

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Six new R2 homologues respond to PiAVR2/PEXRD11 and confer resistance to *P. infestans*.

To test the function of the 17 new *R2* variants, we cloned them under *Rpi-blb3* promoter and terminator, and transferred them to *Agrobacterium tumefaciens* strain AGL1. We coinfiltrated *N. benthamiana* leaves with mixtures of the *A. tumefaciens* strains expressing the *R2* variants with either *PexRD11* (21-1), *PexRD11* (43-1), or *PiAvr2*, and we visually scored the symptoms at six days post infiltration. Six new variants (ednR2GH1, snkR2GH3, snkR2GH4, hjtR2GH2, hjtR2GH3, and hjtR2GH5) induced cell death when co-expressed with PiAVR2 and PEXRD11 (21-1) (Figure 7). These six R2 homologues shared between 92.1 and 99.9% identity, at the amino acid level, with R2, R2-like, Rpi-abpt, or Rpi-blb3 (Table 4), and the six clustered in either clade I or II. No cell death was observed for any member of clades III to VI. Again, cell death with all clade I and II members was more severe when triggered by PiAVR2 than by PEXRD11 (21-1). HjtR2GH5 showed a severe response to PEXRD11 (21-1). A low degree of auto-necrosis induction, however, was visible when hjtR2GH5 was expressed alone (Figure 7).

In order to test the extent to which the *R2* variants conferred resistance to *P. infestans*, we conducted transient complementation assays in *N. benthamiana* (Lokossou et al. 2009). The same six *R2* homologues (*ednR2GH1*, *snkR2GH3*, *snkR2GH4*, *hjtR2GH2*, *hjtR2GH3* and *hjtR2GH5*) that responded to the effectors also conferred specific resistance to *P. infestans* isolate IPO-0 with visible HR (Figure 8) but not to IPO-C, and the six were designated *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*. As expected, based on the effector infiltrations, *snkR2GH2* belonging to clade III, *ednR2GH3* belonging to clade V, and *hjtR2GH4* belonging to clade VI, were non-functional, and large expanding necrotic lesions surrounded by a sporulation zone were observed on inoculated leaves with the virulent (IPO-C) and avirulent (IPO-0) isolates (Figure 8).

In summary, we showed that the polyploid Mexican *Solanum* species contained multiple *R2* homologues that conferred *R2*-specific resistance to *P. infestans*. Hjt17717-3 contains four functional homologues, *R2-like*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*; the last three are expressed in leaves because they were cloned from a leaf tissue cDNA. Snk498042-1 and edn607474-3 each contain two functional *R2* homologues, *Rpi-snk1.1* and *Rpi-snk1.2* for snk498042-1, and *R2-like* and *Rpi-edn1.1* for edn607474-3.

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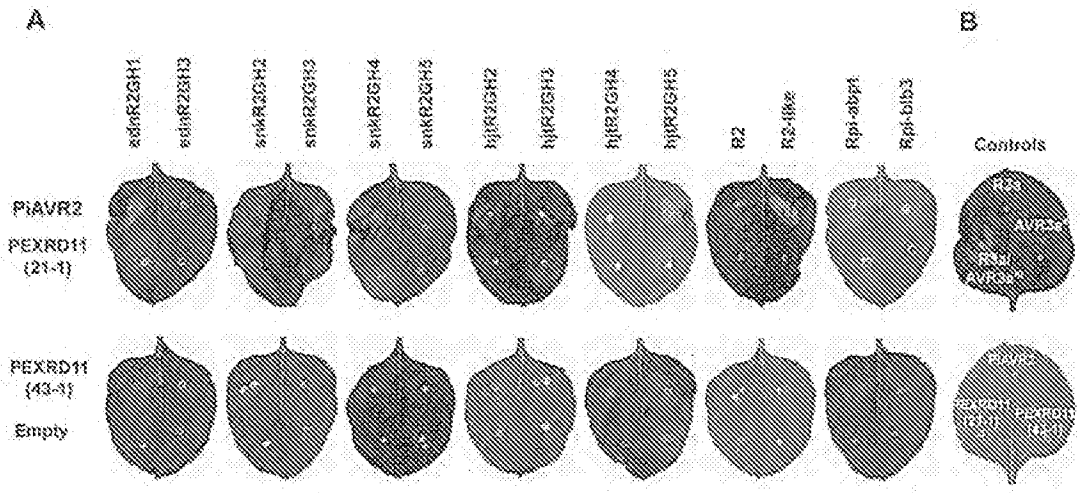


Figure 7. PiAvr2 and PexRD11 induce cell death with the six new *R2* variants.
(A) *Agrobacterium*-mediated expression of PiAvr2, PexRD11 (21-1) and PexRD11 (43-1) with the ten class I and II R2 variants and one representative of the class III to VI in *N. benthamiana*. Infiltration of each *R* gene alone was performed to test the R protein stability. (B) Other controls include the co-infiltration of *R3a*/*Avr3a*^{KI} as positive control and single infiltration of all effector clones used as negative controls.

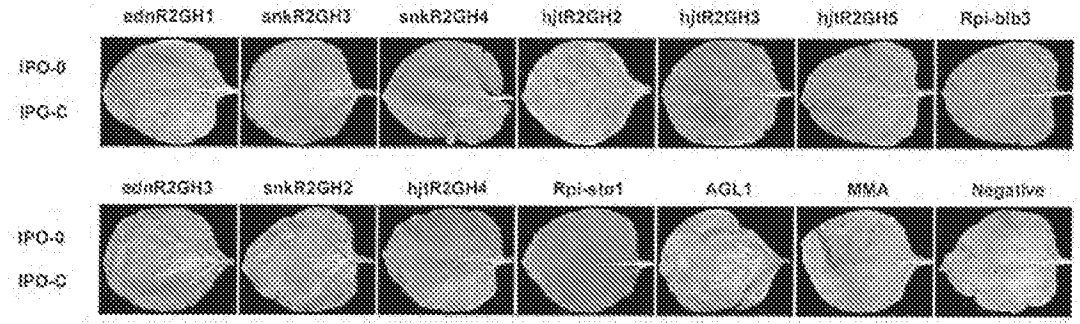


Figure 8. The six new *R2* variants confer resistance to *Phytophthora infestans*.
Transient expression of *R2* variants in *N. benthamiana*, followed by inoculation with the avirulent *P. infestans* isolate IPO-0, and the virulent isolate IPO-C on R2-plants. The six new *R2* homologues, *Rpi-blb3*, and three *R2* variants (*edmR2GH3*, *smkR2GH2* and *hjtR2GH4*) from clade III, V and VI are presented. *Rpi-sto1* from *S. stoloniferum* was included as a resistant control (Vleeshouwers et al. 2008). *N. benthamiana* leaves infiltrated with medium (MMA), with *A tumefaciens* strain AGL1, or not infiltrated were used as negative controls. HR was observed only with all six new *R2* homologues and *Rpi-sto1* when challenged with IPO-0.

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Discussion

With the availability of genome sequences and genetic mapping information for numerous resistance loci in plants, the main challenge now is to identify functional *R* genes among candidate RGHs. In this paper, we rapidly and drastically reduced the number of R2GH to analyze by combining *R2* allele-mining with PEXRD11 effector assays. From a *Petota* selection-wide pool of 100 species originating from North, Central, and South America, we found that R2 activity was geographically restricted to Mexican species. We pinpointed three species (*S. edinense*, *S. schenckii* and *S. hjertingii*) bearing six *R2* homologues (*Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*) that induced cell death with PiAVR2 (Lokossou et al. 2009) and PEXRD11, and conferred resistance against *P. infestans*. We demonstrated that *R2* family is under diversifying selection and identified putative *R* gene domains that might be the target for adaptive evolution and recognition with cognate effectors PiAVR2 and PEXRD11, or their effector target.

Genetic mapping of the gene responding to PEXRD11 in *S. edinense* and *S. schenckii* led us to the *R2* cluster on potato LG IV. From this locus, *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* were recently cloned and the four gene products recognized the RXLR effector PiAVR2 (Lokossou et al. 2009). These R2 homologues originate from various *Solanum* germplasms, including breeding line AM3778-16. In our study, AM3778-16 showed cell death response to PEXRD11 and PiAVR2, and we demonstrated that *S. edinense*, *S. schenckii*, and *S. hjertingii* also responded to PiAVR2. Thus, similar to some other R genes, such as *Rpi-blb1*, *Rpi-blb2* or *Gpa2* which recognize multiple effector variants (Champouret et al. 2009; Oh et al. 2009; Sacco et al. 2009), *R2* homologues responded to at least two effectors, *PiAvr2* and *PexRD11*. Although the overall sequence similarity between PiAVR2 and PEXRD11 was low (23%), a protein structure of 63% similarity emerged in the C-terminal domain, which is often dedicated to effector function and is a target of positive selection (Win et al. 2007). These observations suggest that interaction between PiAVR2/PEXRD11 effectors of *P. infestans* with similar motifs in their C-terminal domain might occur with *R2* homologues and follow the gene-for-gene or the guard hypothesis model (Flor 1971; Jones and Dangl 2006).

Evolutionary analysis of the *R2* sequences suggested that 41 residues are under selective pressure and distributed in LRR domains, particularly in repeats 8, 10, 11, 12 and 13, in L-zipper, and NB domain, but not in the ARC domains. These results are in line with the different current models of R/AVR interaction (Collier and Moffett 2009; Moffett 2009; Takken and Tameling 2009). The R protein activation model of Takken and Tameling (2009) characterizes the activation for signal transduction of an R protein by ATP/ADP binding in the NB-ARC domain, and more precisely in the MHDV motif, which is thought to be analogous to the sensor II motif of AAA+ ATPases (Takken et al. 2006). This activation model would explain why positive selection was not observed in ARC domains. Collier and

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Moffett (Collier and Moffett 2009) proposed a two-step recognition process involving interactions with both cellular cofactors (guardees) and the LRR domain, which in turn activates the molecular "switch" leading to resistance. This process of recognition would explain the identification of residues under positive selection in the L-zipper and NB domains of *R2*, perhaps for the guard function of the cofactor, as well as in the LRR domains to function in effector recognition. There are examples that support this model, including the ternary interactions of p50/NRIP1/N and Gp-RBP-1/RanGAP2/Gpa2. Here different variants of the effectors interact with corresponding cofactors that are guarded by R proteins. The region that determines whether resistance response is executed, however, is under control of LRR domains (Caplan et al. 2008; Sacco et al. 2009; Ueda et al. 2006). Regarding the R2 family, ten of the 27 residues identified as under positive selection pressure were solvent-exposed residues within the LRR repeats 8, 10, 11, 12 and 13 (Figure 6), consistent with their putative function for interaction with potential ligands (Wulff et al. 2009; Zhou et al. 2006). Domain swapping between LRR repeats 11, 12, and 13 of functional and non-functional variants of R2 from Ma-R2 plant has shown the importance of these three repeats in PiAVR2 recognition (Lokossou 2010). Subsequent domain swapping or point mutations in the L-zipper, NB, and more LRR domains of *R2* genes promises to identify which sites are involved in effector recognition, guarding function, or molecular "switch".

Solanum section *Petota* is known to represent a great and diverse resource of *R* genes to *P. infestans*, and functional studies with the cognate *Avr* genes shed light on the evolution of the *R2* gene family. Most *Petota* species occur in the Andes of South America, and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). Hawkes (1990), postulated that the ancestral potato species originated from Central America, then migrated to South America, and returned to Mexico. This migration was followed by hybridization and allo-polyploidisations within Central American taxa, which then led to species with various ploidy levels. We found the functional activity to PEXRD11 and PiAVR2 to be geographically restricted to Mexico, which is an eminent source of *R* genes against *P. Infestans* (Ballvora et al. 2002; Black and Gallegly 1957; Huang et al. 2005; Lokossou et al. 2009; Malcolmson and Black 1966; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Vleeshouwers et al. 2008).

Regarding the broader family of *R2*, we identified a diverse set of 17 new variants and observed a robust association between phylogenetic relatedness and biological function, because cell-death inducing activity with *PiAvr2* and *R2*-specific resistance to *P. infestans* was restricted to clade I and II. The 11 variants of *R2* from clade III to VI showed more divergence compared with *R2* homologues. Because some of these *R2* homologues are transcribed, we can postulate that they are functional genes against other effectors of *P. infestans* or other pathogens. All species exhibiting functional *R2* homologues, i.e. *S. edinense*, *S. schenckii*, *S. hjertingii*, *S. bulbocastanum*, and *S. demissum* (Lokossou et al. 2009), are from Mexico, therefore, we postulate that the *R2* potato blight resistance family

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emerged in Mexcico, one of the centres of origin of the pathogen (Grünwald and Flier 2005). The cloning of a QTL *Rpi-mcd1* in the short arm of LG IV of the South American species *S. microdontum* (Lokossou 2010; Tan et al. 2008) might provide further information on functional aspects of the diverse *R2* clades related to geographical co-evolution with local *P. infestans* genotypes.

The origin of *R2-like* in the AM3778-16 breeding material (Lokossou et al. 2009; Park et al. 2005c) is elucidated in this study, because *R2-like* was amplified from *S. edinense*, which is one of the progenitors in the AM breeding material. Occurrence of identical sequences between species was identified in three cases, which could be due to interspecific hybridization events rather than to ancestral origin of these phylogenetically distant species (Jacobs et al. 2008). That *S. edinense* is a hybrid between *S. demissum* and *S. tuberosum*, and that *R2* originates from *S. demissum*, supports this hypothesis (Li et al. 1998; Serquen and Hanneman 2002).

Recent studies on *Rpi-blb1* have shown that increasing the copy number or transcript level of an *R* gene in transgenic plants improves level of resistance against *P. infestans* (Bradeen et al. 2009; Kramer et al. 2009; Kuhl et al. 2007). There is, however, contradictory data on fitness costs of *R* genes in transgenic plants (Bergelson et al. 2001; Goss and Bergelson 2007; Kniskern and Rausher 2006; Korves and Bergelson 2004; Tian et al. 2003), and recent study revising the work of Tian et al. (2003) demonstrates that their observation on *Arabidopsis thaliana* remained inconclusive (Schwachtje et al. 2008). In our study on natural germplasm, we found two copies of functional *R2* homologues present in *S. edinense*, two in *S. schenckii*, and four in *S. hjertingii*, most likely due to their penta-, hexa-, and tetraploid background; no aberrant phenotypes were observed on any of the three wild species. Our work highlights the importance of late blight resistance in natural populations of *Solanum*. These *Solanum* genotypes are naturally exposed to a high degree of genetic diversity of *P. infestans* population originating, from the commercial potato-growing fields, and the native *Solanum* spp. on the forested slopes in the centre of origin of the pathogen (Flier et al. 2003). The high degree of natural diversity in functional late blight resistance genes is a boon for potato breeders, and offers a number of new genes and traits that can be translated to commercial agriculture.

In summary, the identification of large numbers of *R2* variants in Lokossou et al. (2009) and in this study illustrates that the *R2* family is highly diverse in *Solanum*. The demonstrated *R* gene diversity, such as in the flax rust *R* genes (Dodds et al. 2006) and the *Arabidopsis RPP* genes (Allen et al. 2008; Hall et al. 2009; Rehmany et al. 2005), matches a "use-it-and-diversify-it" R-AVR co-evolutionary dynamics model, in which both R and AVR display high diversification and differential recognition. It will be of interest to identify additional *PiAvr2* family members within the *P. infestans* genome, and in contemporary populations, and test their functionality for different effector targets in the host, to gain

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further insight in recognition events that test the guard or decoy models (Dangl and Jones 2001; van der Hoorn and Kamoun 2008).

Materials and Methods

Plant material

The wild *Solanum* plant material used for effector screening and functional allele-mining is listed in Table 1 and Table 2, respectively. The accessions were retrieved from Centre for Genetic Resources, the Netherlands (CGN), from PI: NRSP-6 – United States Potato Genebank), and from CPC: Commonwealth Potato Collection, UK.). Two populations were generated for genetic studies. Edn607474-3 population was originated from the cross between *S. edinense* (PI: 607474-3) and potato cultivar Concurrent. Snk498042-1 was originated from *S. schenckii* (PI: 498042-1) with *S. stoloniferum* (CPC: 0012-1). Plant genotypes were maintained *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18 °C. Top shoots were transferred to fresh medium; two weeks later, rooted plantlets were transferred to the greenhouse. *Nicotiana benthamiana* plants were grown from seeds in the greenhouse and subjected to agroinfiltration experiments in climate chambers at an ambient temperature of 22–25 °C and high light intensity.

Phytophthora infestans isolates and disease tests

The *P. infestans* isolates IPO-0 (race 3b.4.7.10.11), 90128 (race 1.3.4.7.8.10.11) and IPO-C (race 1.2.3.4.5.6.7.10.11) (Champouret et al. 2009) were cultured on rye sucrose agar medium in the dark at 15 °C for 1 to 2 weeks (Caten and Jinks 1968). Sporulating mycelium was flooded with cold water, and the sporangiospore suspension was incubated at 4 °C for 3 h. After the release of the zoospores, the inoculum was adjusted to a concentration of $5*10^4$ spores/ml. Leaves from 6 to 8 week-old plants were detached and placed in water-saturated oasis in humid trays; leaflets were spot-inoculated with the spore suspension. The trays were incubated in a climate chamber with a 16h light and 8h dark cycle at 15 °C. At six days post inoculation, lesion sizes (LS) were measured. Sporulating lesions exceeding 15 mm were scored as susceptible (S), whereas lesions displaying localized hypersensitive response (HR) smaller than 5 mm at the inoculation sites were scored as resistant (R). For high throughput resistance screening in *Solanum* germplasm, disease tests were performed in field trials, in detached leaf, and in vitro assays. Scores were assessed and converted to a relative scale of 9 to 1. Nine corresponds to no symptoms or very small LS due to localized HR, 2 is set to the susceptible standard Bintje, 1 is massively sporulating and large lesions exceeding those of Bintje, and the other ratings correspond to intermediate classes, as shown on the *SolRgene* database website (*SolR*gene database).

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PCR allele mining

R2 variants from wild *Solanum* species were cloned by PCR amplification on genomic DNA, using the conserved start and stop primer pair blb3-F-Start-GW and blb3-R-stop-GW (Table 6) containing AttB1 and AttB2 sequences for Gateway cloning purposes. Amplicons were cloned into pDON221. For amplification on *S. hjertingii*, mRNA was extracted from hjt17717-3 leaves using RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN), and cDNA was made using iScript™ cDNA synthesis kit (Biorad). Amplification was made using Phusion High-Fidelity DNA Polymerase (FINNZYMES). PCR fragments were purified on 1% TBE gel using Min Elute™ Gel Extraction Kit (QIAGEN) and were cloned in pDONR221 (INVITROGEN) using BP clonase (INVITROGEN). Primers were designed on *R2* (Table 6) to sequence clones using Greenomics facilities (Wageningen, The Netherlands), and sequences were analyzed using DNASTAR Lasergene v8. Clones containing a *R2* variant in frame were cloned under *Rpi-blb3* promoter and terminator using multisite Gateway (Lokossou et al. 2009).

Table 6: Primers list.

Primer name	Primer sequence 5'- 3'	Comments
RGH-SSR51F	CGCAGTATACGCTGTCCAAC	SSR marker RGH-SSR51
RGH-SSR51R	GTTTGCAGTGTGAGTTGGTTGGTG	
Th21F	AGCATCCGGAGGCAAAATC	HRM marker
Th21R	TAGGCTTAACTGTCAAATGG	
R2ch4F	TGTGCAGTGATAACAGCTTCA	R2 NBS marker
blb3-F-start-GW	GGGGACAAGTTTgtacaaaaaagcaggctATGGCTGATGCCTTTCTATCATTTG	R2 variants amplification
blb3-R-stop-GW	GGGGACCACTTTGTACAAGaaagctgggrTCACAACATATAATTCCGCTTCAAC	
R2F	ATGGCTGATGCCTTTCTATCATTTGC	
SpF2	GAAGGAGAAGAAATTCTACAATG	
Glo2F	GTGTCTCTCAAGAGTACAACAC	
SpF4	TAATACGGTTGTGGATGGCG	
abptF2	CAAGAAGCCACTCCATATCC	
Lrr-F1	CTCTTTATGTATCAGACATGGC	
SpF11	CCGTCTTCCATTGGCAACC	
SpR1b	CATTGTAGAATTTCTTCTCCTTC	
SpblbR	ACTTTTTCCCAAAATGTTTTAGC	
SpR3	AGCTCCTGATACGATCCATG	
LrrR3	CAACATCTTTCCACTGATCAC	Sequencing
abptR3	TCAACAGGGTCAACATCTTTC	
LrrR2	GAGGCATGGCACTTGTGCC	
R2R	TCACAACATATAATTCCGCTTC	
R2-HJT	GGAAACTACTTGATGTTCGAG	
R2-HJT-NC57_17F532	AAATCTCTTCAGGCTGAGGC	
R2-HJT-NC57_17R1	TGCCATACATCATCAACCAC	
R2-HJT-NC57_17F682	CTCTGGACAAGAGTTGAGTC	
R2-HJT-NC57_17R2	CTTGGCGCTGCCTTGGATG	

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Agroinfection and Agroinfiltration

A collection of 55 non-redundant RXLR effectors was identified in previous studies, and primer pairs based on the mature region of candidate effectors were designed and used to amplify total DNA from a panel of nine *P. infestans* isolates (Oh et al. 2009; Vleeshouwers et al. 2008). Amplicons were cloned into the pGR106 (Lu et al. 2003) for occurrence of cell death responses on *Solanum* plants using PVX agroinfection, as described previously (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers and Rietman 2009), and pGR106::empty vector and pGR106::CRN2 were included as negative and positive controls in that order. Each individual effector was tested twice on six leaves over two plants in two independent experiments. Leaves were monitored for symptoms over 16 days.

PexRD11 clone 21-1 (NCBI Genbank code GQ869426) and clone 43-1 (NCBI Genbank code GQ869427), without signal peptide, were introduced in pK7WG2 (Karimi et al. 2002) by gateway technology (see primers used in Table 6). *Avr3a*^{KI} and *PiAvr2* (Lokossou et al. 2009), without signal peptide, were cloned in pGRAB, as was *R3a* (kindly provided by S.N. Chapman). *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) was transformed with individual binary vectors in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000). *Solanum* plants infiltration with *A. tumefaciens* was performed as described (Vleeshouwers and Rietman 2009). The *A. tumefaciens* strains were grown as described (van der Hoorn et al. 2000), except that culturing steps were performed in LB media supplemented with 50 µg/ml kanamycin. Strains were grown to desired optical density at 600 nm (OD_{600}) of 0.2, although the same results were observed with OD_{600} between 0.1 to 0.4. *A. tumefaciens* solutions were mixed in a 1:1 ratio for co-infiltration. Leaf panels of middle age leaves on *Solanum* plants beyond nine-leaf stage, or four- to five-week-old *Nicotiana benthamiana* plants were infiltrated with the *A. tumefaciens* suspensions, and symptoms were monitored from two to six days post infiltration (dpi).

Genetic mapping

The SRR marker RGH-SSR51 (Table 6) was developed by Dr. A. Goverse (Laboratory of Nematology, Wageningen University, The Netherlands). PCRs were performed using the following cycle profile: an initial cycle at 95 °C for 2 min; then 30 cycles of (i) 95 °C for 30s sec, (ii) 56 °C for 30 sec, using a ramp of 1 °C/min, and (iii) 72 °C for 45 sec, using a ramp of 1 °C/min; and a final step at 72 °C for 3 min. PCR products were analysed by electrophoresis acrylamide gels on the LI-COR sequencer (Lincoln, Nebrasca, U.S.A.). SCAR marker Th21 primer (Table 6) combination (Park et al. 2005b) was tested with the High Resolution Melting curve analysis (HRM) on the LightScanner (Idaho Technology). PCRs were performed using the following cycle profile: an initial cycle at 98 °C for 30 sec then 40 cycles of three steps, (i) 98 °C for 5 sec, (ii) 60 °C for 5 sec, and (iii) 72 °C for 15 sec; followed by three final steps of 72 °C for 30 sec, 94 °C for 30 sec, and 25 °C for 30 sec. The *R2* PCR marker R2-2350-Rsa was developed by NBS profiling (van der Linden et al.

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2004), and resulted from the primer R2ch4F4 (Table 6) in combination with the restriction enzyme RsaI.

Transient complementation in *N. benthamiana*

Four-week-old *N. benthamiana* plants were infiltrated using an OD_{600} of 0.2 with a solution of *A. tumefaciens* AGL1 (Lazo et al. 1991) harbouring the putative *R* gene candidates. Two days after infiltration, a detached leaf assay was performed with *P. infestans* isolates IPO-0 and IPO-C, symptoms were scored (Lokossou et al. 2009) between four and eight days post inoculation.

Positive selection analysis

To test for amino acids under purifying or diversifying selection, we used codon-based analysis (Codeml) implemented in PAML v.4.2 package (Yang 2007). Maximum-likelihood codon substitution models M0, M1, M2, M7, and M8 were used for analysis (Win et al. 2007). Positively selected sites detected by, models M2 and M8 were identified using Bayes Empirical Bayes statistics (Yang et al. 2005).

Phylogenetic data analyses

Phylogenetic analyses of the new R2 variants from the wild species were conducted using the Neighbor-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). R2, R2-like, Rpi-abpt, Rpi-blb3, and RPP13 protein sequences were used as closest known homologue genes, and the tree was rooted using R3a. Bootstrap values greater than 60% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by MEGA. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences (Tamura et al. 2007).

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DNA sequence data is available in the NCBI GenBank database under the accession numbers GU563963 through GU563979

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CHAPTER 4

Diversity of *PiAvr2/PexRD11* and *R2* gene families underpins co-evolution between *Phytophthora infestans* and Mexican *Solanum* species

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In preparation for submission

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Diversity of *PiAvr2/PexRD11* and *R2* gene families underpins co-evolution between *Phytophthora infestans* and Mexican *Solanum* species

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Abstract

The interaction between potato and the late blight pathogen *Phytophthora infestans* is controlled in a gene-for-gene manner by cytoplasmic resistance (*R*) genes of the CC-NBS-LRR group, which confer recognition of their cognate pathogen avirulence (*Avr*) proteins. R2 is one of these *R* genes. It recognizes two effector proteins of *P. infestans* PiAVR2 and PEXRD11. In this study, we focussed on the identification and characterization of *PiAvr2/PexRD11* family members within the *P. infestans* genome. We performed BLAST similarity searches of the genome sequence of *P. infestans* with the C-terminal effector domain of PiAVR2 and PexRD11 and identified 11 independent RXLR effectors that were grouped in classes I, II and III. Robust association between phylogenetic classes of the *PiAvr2/PexRD11* effector family and *R2* family with recognition leading to cell death was uncovered. The ten known functional R2 homologues recognised three new effectors with different specificities. One effector protein, PITG_13940 induced cell death with the six R2 homologues, i.e. Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3, but not with R2, R2-like, Rpi-abpt and Rpi-blb3 which were used in breeding material for the last decades. Interestingly escape of recognition of PITG_13940 is linked to two amino acids in the C-terminal domain. Two other effectors, PITG_14936 and PITG_05121, from the class I and II did not induce hypersensitive response (HR) with R2 homologues, and different hypotheses based on secondary structure, presence of a KIT/KIK/PIK motif and folding protein arrangement are presented. Also, we predicted that PiAVR2/PEXRD11 family members of class I and II contain a Nuclear Export Signal (NES) and that the presence of the

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potative NES sequence is linked to the induction of cell death with R2 homologues. All together, these results highlight the first functional support for the arms-race model in the *Solanum*/*P. infestans* pathosystem based on the R2-PiAVR2/PEXRD11 interaction. Moreover using effectoromics we could characterize six new *R2* homologues variants with potentially broader resistance spectrum. This study, therefore, provides more *R* gene diversity and specificity, valuable for potato breeders.

Introduction

Plant diseases impose a major constraint on food production worldwide. They are often combated by chemical, genetic or biological methods. Nowadays, the management of plant diseases by chemical approaches is becoming more and more unpopular even if in general it is a reliable process. An emerging interest for a more economical and sustainable crop production system in concert with the protection of the environment renders genetic methods highly desirable. Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is a huge threat for potato and tomato farming. Chemical management of the disease has been relatively successful, however, developed resistance to the main fungicide, the phenylamide metalaxyl (Cohen and Reuveni 1983; Cooke 1981; Davidse et al. 1981; Deahl et al. 2002; Deahl et al. 1993; Miller et al. 1997), has led to new interest in exploring the wealth of Resistance (*R*) gene diversity in tuber-bearing *Solanum* species of the section *Petota*. In those plants, new *Resistance* genes to *P. infestans* (*Rpi*), with different spectra of resistance were discovered and cloned (Foster et al. 2009; Kuhl et al. 2001; Lokossou et al. 2009; Pel et al. 2009; Sandbrink et al. 2000; Smilde et al. 2005; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Villamon et al. 2005; Vleeshouwers et al. 2008). Interestingly all these studies have brought more insight in *R* gene diversity from different *R* gene clusters, by identifying several *R* genes homologues (*RGH*) in various wild *Solanum* species (Lokossou et al. 2009; Pel et al. 2009); This thesis Chapter 3). From the R2 cluster, which was studied by map based cloning and functional allele mining, up to 50 non-redundant *R2* variants were isolated from *S. demissum*, *S. bulbocastanum*, *S. edinense*, *S. schenckii*, and *S. hjertingii*, with ten of them being functionally active against late blight (Lokossou et al. 2009; Chapter 3). Interestingly, these *R2* homologues are responding to two RXLR effectors of *P. infestans*, *PiAvr2* and *PexRD11*, which have a low percentage of identity sensu stricto but a 63% of similarity based on identical, conserved and semi-conserved amino acid (a.a.) substitutions (Chapter 3).

In a plant-pathogen co-evolutionary state, the extreme intimacy between plant and pathogen drives a complex and dynamic selection pressure on the genes involved in the interaction. Adaptation of the host by gain of resistance (via an *R* gene), changes the selection pressure on the pathogen, giving rise to a counter-adaptation. If this occurs reciprocally

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(escape of recognition by mutation or silencing of the avirulence gene and therefore gain of virulence on the host), an unstable runaway escalation or "arms-race" occurs. These produce an up/down effect in the interaction, visible by a cycle of resistance followed by susceptibility, which is described as a Zig-Zag model by Jones and Dangl (2006). Effectors in that case act as avirulent factors (*Avr*) and their recognition by *R* genes is based on the gene-for-gene model (Flor 1971). Examples of such direct interaction are relatively rare (Deslandes et al. 2003; Dodds et al. 2006; Ellis et al. 2007a; Jia et al. 2000). Van der Biezen and Jones (1998), have come up with a new model, the guard model, in which the R proteins do not directly detect the presence of the pathogen avirulence protein, but perceive alterations of the virulence target protein (guardee) by guarding it for interaction with the effector. This model is well illustrated with effector proteins from *Pseudomonas syringae* and R proteins of *Arabidopsis thaliana* (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002; Shao et al. 2003; van der Linden et al. 2004).Yet recognition specificity does not always correlate with virulence activity. van der Hoorn and Kamoun (2008), therefore, came with a decoy model. In this model of evolution, guardees are subject to alternating selective pressures depending on the presence or absence of their guarding R protein. In the presence of the R protein, the guardee would be optimized for Avr interaction, and hence detection. In the absence of the R protein, the guardee would be under pressure to evade interaction with pathogen effectors in order to reduce pathogen virulence. The recent completion of the *P. infestans* genome (Haas et al. 2009) brings new opportunities to study the evolution of gene families of this pathogen. In a previous study we have postulated that the *R2* gene family emerged in Mexico (Chapter 3), one of the centres of origin of the pathogen (Grünwald and Flier 2005). In this respect, the interaction between Mexican *Solanum* species and *P. infestans* provides an excellent opportunity for functional and evolutionary studies of *R2* – *PiAvr2/PexRD11* family interaction.

In this chapter, we examine the pattern of gene diversity of the *PiAvr2/PexRD11* family using the recently sequenced *P. infestans* genome of strain T30-4 (Haas et al. 2009). In total 13 different RXLR secreted proteins of the *PiAvr2/PexRD11* family were identified, and we tested their avirulence against 21 R2 variants originating from five Mexican *Solanum* species (Chapter 3). Our results show a robust association, with some differential specificity, between phylogenetic classification of the *R2* variants and members of the *PiAvr2/PexRD11* family. We also provide functional evidence of the co-evolution between the *R2* family and the cognate *PiAvr2/PexRD11* family.

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Results

***PiAvr2/PexRD11* effector family is highly diverse.**

To identify specificity of recognition between *P. infestans* effectors and *R2* homologues, we first mined the *P. infestans* T30-4 genome for effectors with homology to *PiAvr2* and *PexRD11*. In a previous study we demonstrated that *PexRD11* has a functional redundancy with *PiAvr2* by being recognized by functional *R2* homologues of different *Solanum* genotypes. Although the overall sequence similarity between PiAVR2 and PEXRD11 is low (23%), a protein structure similarity of 63% emerged in the C-terminal domain based on identical, conserved and semi-conserved amino acids substitutions. A BLASTp search of the *P. infestans* T30-4 genome sequence database with the C-terminal domain of PiAVR2 and PEXRD11 resulted in the identification of 21 independent gene models, 13 out of 21 come from a BLASTp search using PiAVR2 as query and eight from BLASTp search using PEXRD11 as query. In total, 18 out of the 21 gene models when translated to proteins annotated as secreted RXLRs (Table 1). Gene sequence analyses of the 18 RXLR effectors revealed that *PiAvr2* and *PITG_22870*, *PITG_08278* and *PITG_20025*, *PITG21645* and *PITG_13956*, *PITG_23009* and *PITG_23008* are present as double copies in the T30-4 genome. PITG_21645 and PITG_23008, which have three amino acid differences between the signal peptide and the EER motif, are fully identical in their C-terminal domain, and therefore represent only one variant in this selection. The two BLASTp searches show very different homologous proteins with only one protein hit in common PITG_21949. Finally, we identified 11 non-redundant effectors in *P. infestans* T30-4 genome having some degree of similarity with the C-terminal domain of PiAVR2 and PEXRD11 (Table 2).

Neighbour-joining phylogenetic clustering of the C-terminal domain of 13 PiAVR2/PEXRD11 family members resulted in three classes that were designated with the suffix I, II, and III (Figure 1). Class I contains PEXRD11 and four other effectors (PITG_13940, _21949, _21645 and _13936) which are all from the RxLRfam7 previously described by Haas et al. (2009), and have a 60 to 62.6% similarity with PEXRD11 (Table 2). Class II contains PiAVR2 and PITG_05121 with 43% of similarity. Class III contains the six remaining effectors which are more divergent with only 30.4 to 41.3% of similarity to PEXRD11 and PiAVR2 (Table 2).

Specificities of recognition of R2 homologues with class I and II PiAVR2/PEXRD11 family effectors.

In previous studies, we showed that *PexRD11* and *PiAvr2* specifically trigger an *R2*-mediated cell death with ten homologues from *S. edinense*, *S. schenckii*, *S. hjertingii*, *S. demissum*, *S. bulbocastanum* and two breeding clones, but not with 11 other *R2* variants (Chapter 3). To assess whether the newly identified *PiAvr2/PexRD11* family members are also recognized by *R2* homologues, their C-terminal domain was synthesized and inserted in

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Table 1: List of genes in *Phytophthora infestans* genome T30-4 with sequence similarity to the C-terminal effector domain of PiAVR2 and PEXRD11.

A

Gene Name	Genome location	Gene description	Score (Bits)	E-value	Alignment length	Identities in percentage (%)	Positives
PITG_22870	Supercontig 16: 1903483-1903843	Secreted RxLR effector peptide, putative	117.6	$4.99e^{-26}$	100	100	100
PiAvr2 (PITG_08943)	Supercontig 16: 1901141-1901491	Secreted RxLR effector peptide, putative	117.6	$4.99e^{-26}$	100	100	100
PITG_05121	Supercontig 7: 459447-459788	Secreted RxLR effector peptide, putative	43.1	$1.3e^{-5}$	80.4	41.2	51
PITG_19617	Supercontig 99: 380469-380845	Secreted RxLR effector peptide, putative	40	$1.13e^{-4}$	64.7	33.3	47.1
PITG_07500	Supercontig 11: 1294260-1294616	Secreted RxLR effector peptide, putative	38.1	$2.1e^{-4}$	64.7	31.4	49
PITG_07499	Supercontig 11: 1290401-1290757	Secreted RxLR effector peptide, putative	38.1	$2.1e^{-4}$	64.7	31.4	49
PITG_08278	Supercontig 13: 612373-612729	Secreted RxLR effector peptide, putative	37.3	$7.23e^{-5}$	64.7	31.4	45.1
PITG_20025	Supercontig 137: 149834-150180	Secreted RxLR effector peptide, putative	37.3	$7.23e^{-4}$	64.7	31.4	45.1
PITG_15972	Supercontig 39: 1376315-1376599	Secreted RxLR effector peptide, putative	36.9	$9.84e^{-4}$	51	33.3	39.2
PITG_16221	Supercontig 43: 623613-625504	Crinkler (CRN) family	28.7	0.138	64.7	27.5	29.4
PITG_00323	Supercontig 1: 1810206-1813748	Conserved hypothetical protein	29.3	0.186	78.4	25.5	47.1
PITG_21949*	Supercontig 811: 3517-3861	Secreted RxLR effector peptide, putative	31.3	0.283	23.5	15.7	17.6
PITG_08877	Supercontig 9: 3123662-3124018	Secreted RxLR effector peptide, putative	28.4	0.348	43	21.6	35.3

B

PexRD11 (PITG_13930)	Supercontig 30: 1248179-1248523	Secreted RxLR effector peptide, putative	121	$4.46e^{-29}$	100	100	100
PITG_21948*	Supercontig 811: 3517-3861	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	100	51	74.5
PITG_21645	Supercontig 550: 43867-44211	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_23889	Supercontig 30: 1532184-1532528	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_23088	Supercontig 30: 1436877-1437221	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_13956	Supercontig 30: 1470702-1471046	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_13940	Supercontig 30: 1349583-1349926	Secreted RxLR effector peptide, putative	63.6	$9.06e^{-12}$	100	47.1	74.5
PITG_13936	Supercontig 30: 1300754-1301044	Secreted RxLR effector peptide, putative	48.2	$1.92e^{-7}$	64.7	39.2	47.1

A BLASTp search using C-terminal effector domain of PiAvr2 (A) and PexRD11 (B) was done on the Broad Institute website (*Sol*Rgene database). () Indicate genes found in both BLASTp search.

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Table 2. Percentage similarity on amino acids level between the 11 new PiAVR2/PEXRD11 family members vs. PiAVR2 and PEXRD11.

Percent of Similarity at the Amino Acid Level			Phylogenetic Class
	PiAVR2	PEXRD11	
PITG_13940	30	60	I
PITG_21949	30	62.6	
PITG_21645	29.1	60	
PITG_13936	34.8	62.9	
PEXRD11	33.6	100	
PITG_05121	43	28.7	II
PiAVR2	100	33.6	
PITG_06077	34.5	30.4	III
PITG_08278	33.6	33.9	
PITG_19617	33.6	33.9	
PITG_15972	41.3	39.1	
PITG_07499	34.5	34.8	
PITG_07500	34.5	35.7	

Percentages of similarity are based on the C-terminal domain of the effector proteins. The three classes described in the phylogenetic tree (Figure 1) are represented on the right.

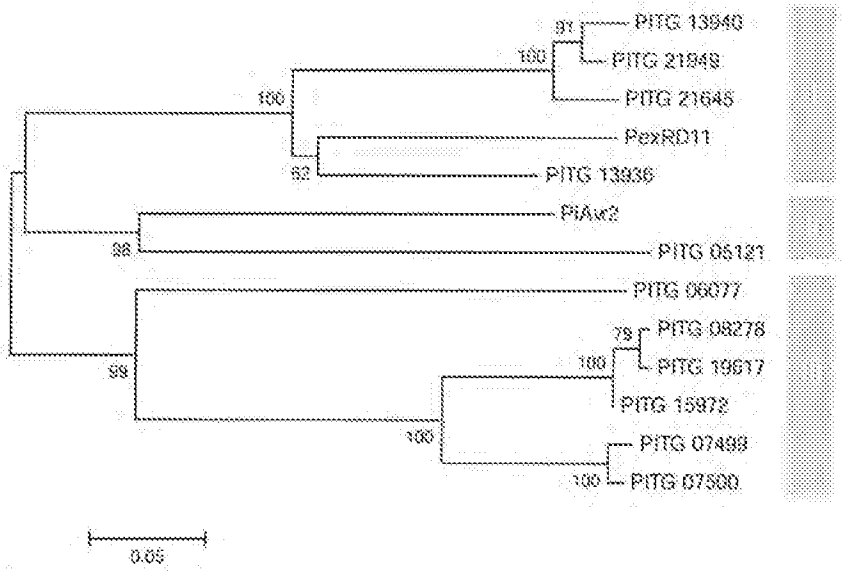


Figure 1. Phylogenetic relationship and clustering of *PiAvr2/PexRD11 family* members resulting in three classes I, II and III.

Neighbour-joining tree illustrating phylogenetic relationship of *PiAvr2/PexRD11 family* members in *P. infestans* strain T30-4. Amino acids sequences are grouped in three classes. Bootstrap values greater than 60% (based on 1000 resample) are indicated at the nodes.

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plasmid pJL-TRBO vector (Lindbo 2007). *Agrobacterium tumefaciens* cultures expressing individual PiAVR2/PEXRD11 members were coinfiltrated with *Agrobacterium* cultures expressing R2 variants in *N. benthamiana* leaves. Four types of responses were observed. The first type of response, observed with PITG_21949 and PITG_21645 (Figure 2), is the typical PiAVR2 avirulence activity that show induced cell death specificity to ten *R2* homologues (Chapter 3). In the second type of response we observed a differential specificity of recognition to PITG_13940. Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3 triggered HR when coinfiltrated with the C-terminal domain of PITG_13940, but no cell death was induced with R2, R2-like, Rpi-abpt and Rpi-blb3. In the third type of response, no cell death with any R2 variant was detected, this correspond to all class III PiAVR2/PEXRD11 members, in addition to PITG_13936 of class I, and PITG_05121 of class II. PITG_13936 is a slightly mutated and truncated form of PEXRD11 and it is present in the PEXRD11 locus (Table 1 and Figure 3). PITG_05121 belongs to the same class as PiAVR2 (Figure 1), and it is located in a different locus (Table 1). Interestingly, PITG_05121 has a unique three amino acid deletion starting after a.a. position 79 which seems relatively conserved among the five functional PiAVR2/PEXRD11 family members and the truncated PITG_13936, a KIT/KIK/PIK motif (Figure 3). The last type of response is described as a high and low degree of auto-necrosis induction, detected for PITG_07499 and PITG_06077 that belong to class III (Figure 2). In conclusion, five members of the PiAVR2/PEXRD11 family were identified to display PiAVR2 activity, of which PITG_13940 showed a differential recognition among *R2* homologues.

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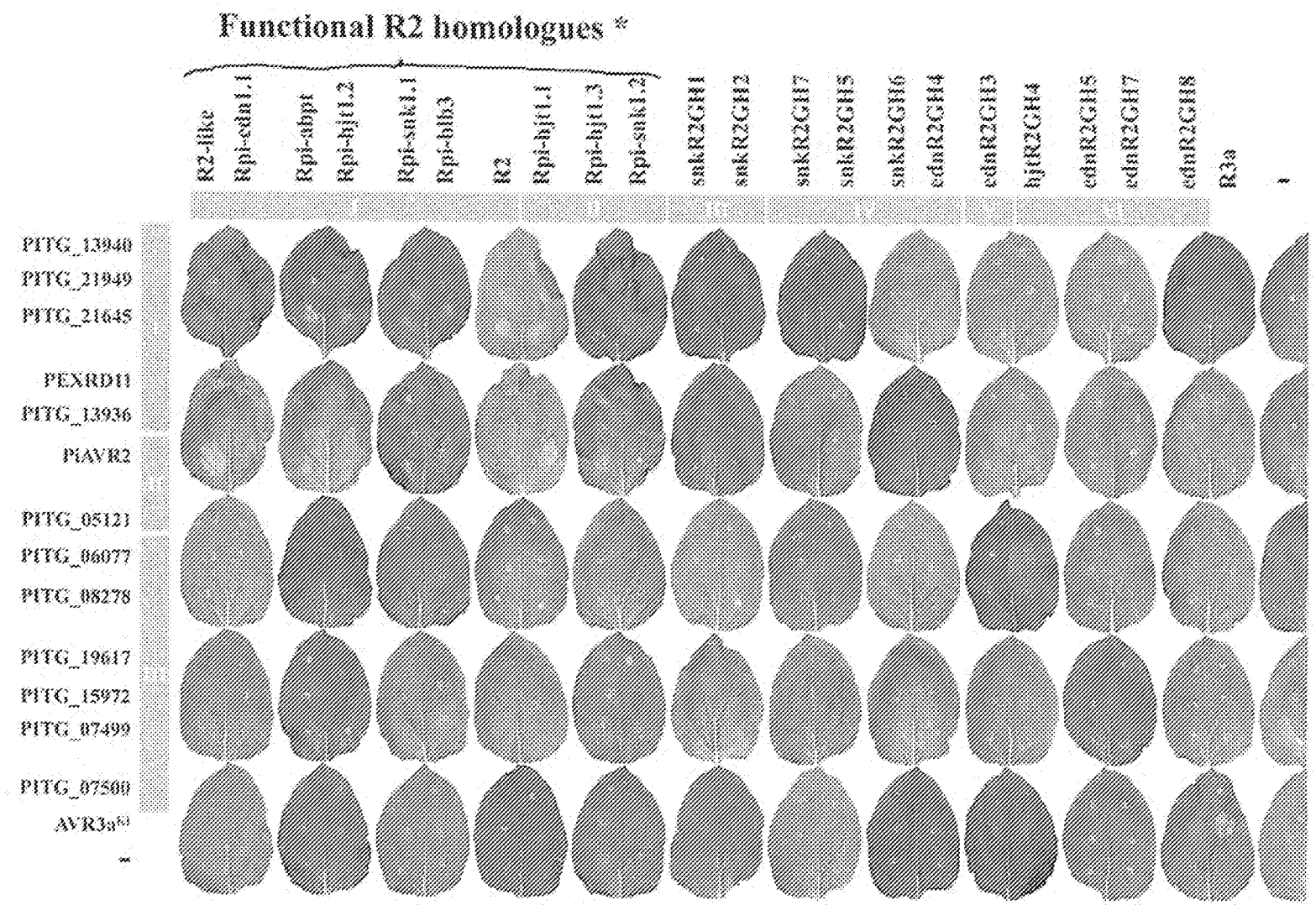


Figure 2. Specificity of recognition between R2 homologues and C-terminal effector domain of the PiAVR2/PEXRD11 family.
Co-infiltration between C-terminal effector domain of PiAVR2/PEXRD11 family members and AVR3a^{KI} versus R2 variants and R3a. The grey blocks on the top of the figure indicate the R2 variants classes as described in Chapter 3, with the ten functional R2 homologues of class I and II. The grey blocks on the left of the figure indicate the PiAVR2/PEXRD11 family member classes as described in Figure 1. (*) Functionality against *P. infestans* demonstrated in Chapter 3. R3a and AVR3a^{KI} were included as controls and each construct was infiltrated alone to detect any auto-necrosis function. Photographs of symptoms were taken at four days post infiltration (dpi).

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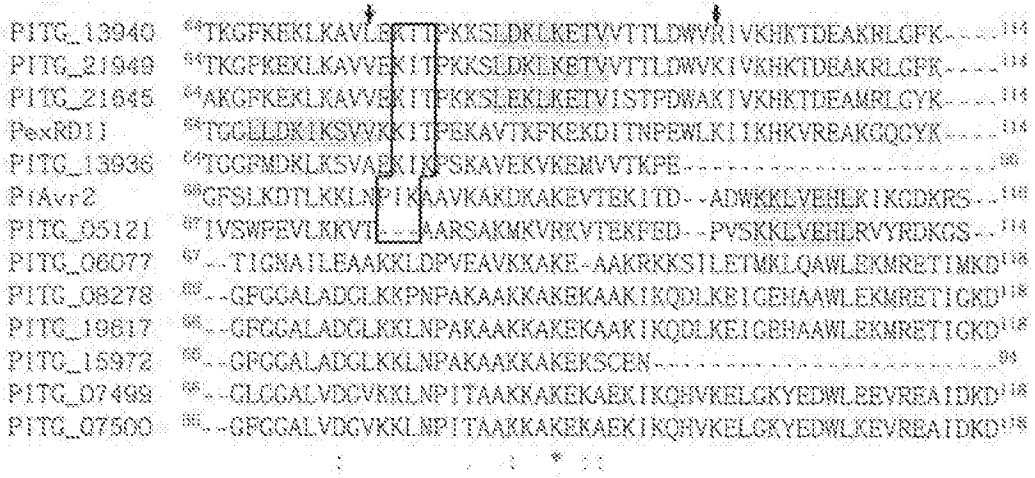


Figure 3. Effectors of PiAVR2/PEXRD11 family recognized by R2 homologues contain a NES motif.
ClustalW alignment of C-terminal effector domain of the 13 effectors from the PiAVR2/PEXRD11 family. (*) Residues in that column are identical in all sequences in the alignment, (:) conserved and (.) semi-conserved substitutions have been observed. Highlighted in grey are the Nuclear Export Signal (NES) predicted by NetNES 1.1 webserver. The Box highlights the KIT/KIK/PIK motif. Arrows pinpoint the difference in two amino acids between PITG_13940 and PITG_21949.

PiAVR2/PEXRD11 proteins recognised by R2 homologues contain a putative Nuclear Export Signal motif (NES)

To investigate the differential response of PITG_13940 among the ten R2 homologues, we compared the C-terminal domain of PITG_13940 and the closest related effector in the PiAVR2/PEXRD11 family, PITG_21949. Only two amino acid changes were identified (Figure 3), i.e. in position 75 a valine is replaced by a leucine residue and in position 99 a lysine is replaced by an arginine in PITG_13940. These amino acid changes could be responsible for structural changes and explain the differential responses between the six Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3 versus R2, R2-like, Rpi-blb3 and Rpi-abpt to recognize PITG_13940.

To determine if the specificity of cell death induced by PITG_21949, PITG_21645, PITG_13940, PEXRD11 and PiAVR2 with the ten R2 homologues was due to the presence of a common motif recognized by the R genes, we aligned the C-terminal domain of all effectors of PiAVR2/PEXRD11 family using ClustalW and searched for any known motif present in each member of the family. No W, Y or L motif typical to *Phytophthora* effectors were identified (Jiang et al. 2008), nor a new motif specific to this family (Figure 3). Interestingly, we identified sequences with similarity to the Nuclear Export Signal motif (NES) in all five effectors with PiAVR2 activity and also in the PITG_05121, which did not trigger HR with any of the R2 variants. Based on this result, we speculated that NES motif might be important for the effector activity of PiAVR2. Since none of the point mutations in PITG_13940 were identified in this NES motif, the escape of recognition of this effector to R2, R2-like, Rpi-abpt and Rpi-blb3 blb3 is probably due to another mechanism, such as

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secondary structure changes which affect its interaction with these R2 homologues or their guardee.

Discussion

With the release of *P. infestans* T30-4 annotated genome (Haas et al. 2009), a wealth of effector genes with positional information became available. In this study, we identified 11 new effectors from *P. infestans* with a C-terminal domain structure related to the recently identified avirulent effectors PiAVR2 and PEXRD11 against *R2* homologues (Lokossou et al. 2009; Chapter 3). Our approach was based on the use of the C-terminal domain of these effectors because it is often dedicated to the effector function and is the target of positive selection (Win et al. 2007). The PiAvr2/PexRD11 variants were grouped in three classes. Interestingly all effectors from the class I and II, except PITG_13936 and PITG_05121, induced HR with different specificity when coinfiltrated with R2 variants from class I and II (Chapter 3). We uncovered, therefore, the first robust association between phylogenetic classes of the *PiAvr2/PexRD11* effector family and *R2* family with recognition leading to HR cell death. These data provide a first functional support for the arms-race model in the *Solanum/P. infestans* pathosystem for the R2-PiAVR2 interaction.

Here we have identified three classes of the PiAVR2/PEXRD11 family, where members of class I/II are triggering cell death in the presence of R2 homologues with different specificity. For three members of class I/II, recognition is partly or fully avoided, for which several evolutionary mechanisms could be involved. (i) Mutation of two amino acids already led to specific escape of recognition of PITG_13940, with the R2, R2-like, Rpi-abpt and Rpi-blb3 proteins. Similar findings were reported for AVR3a of *P. infestans* where the $AVR3a^{KI}$ form is recognised by the resistance gene R3a and the $AVR3a^{EM}$ form is not (Armstrong et al. 2005), but both effector variants can still interact with the same effector target protein, CMPG1, an ubiquitin E3 ligase required for INF1-mediated cell death (Birch et al. 2009; Gonzalez-Lamothe et al. 2006). From a co-evolutionary point of view, this strategy is partially successful for *P. infestans*, since there are only six R2 homologues that recognise PITG_13940. (ii) Truncation of the *Avr* gene leading to recognition avoidance was found for PITG_13936. This mechanism is also described for the *Avr4* effector of *P. infestans*, of which the 'virulent' allele present at the *Avr4* locus encodes a truncated protein, and therefore isolates with the virulent form only can infect Ma-R4 plants (van Poppel et al. 2008; van Poppel et al. 2009). (iii) The third loss of recognition, by PITG_05121 is more puzzling, but perhaps it could be due to a three amino acid deletion, which then has the same mechanism as (i), or the accumulation of 24 mutations (51%) compared to PiAVR2 sequence, has created a new effector with different function for the pathogen. In conclusion, we have identified ten R2 homologues, present in multiple copies in wild *Solanum* species,

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responding to five effectors of the PiAVR2/PEXRD11 family, present in the genome of *P. infestans*, which represent a high number of R/AVR combinations within host/pathogen interaction events, and therefore, demonstrates a real arms-race (Bergelson et al. 2001; Dawkins and Krebs 1979) between potato and *P. infestans*.

At the biochemical/structural level, the specificity of PITG_13940 that caused cell death when co-expressed with six R2 homologues (Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2, and Rpi-hjt1.3), but not with the four other R2 homologues (R2, R2-like, Rpi-abpt and Rpi-blb3) can have two origins. The absence of HR can either be due to a fast recognition inducing Extreme Resistance (ER) response, as between Potato Virus X and Rx (Baurès et al. 2008; Bendahmane et al. 2002; Kang et al. 2005), or to the lack of interaction between PITG_13940 and these four R2 homologues. The failure of HR induction with the four R2 homologues can be explained by various hypotheses. Structural changes of PITG_13940 may prevent direct interaction with the four R2 homologues, or interaction with the guardee or decoy protein, as required for the gene-for-gene (Flor 1971), guard, and decoy models (van der Biezen and Jones 1998; van der Hoorn and Kamoun 2008). An alternative hypothesis is based on the bait and switch model (Collier and Moffett 2009). In that case PITG_13940 can interact with the effector target, but there is no transduction of signal response because the LRR domain of R2, R2-like, Rpi-abpt and Rpi-blb3 do not interact with this effector. On the side of the host R genes, however, we could not identify obvious SNPs that discriminate the six active R2 homologues from the four R2 homologues that did not induce cell death with PITG_13940. Therefors, recognition of PITG_13940 without transduction of resistance signal may be less supported. Future studies on protein folding structure differences between the ten R2 homologues may provide more information. On the side of the effector genes supplied by the pathogen, we identified only two a.a. difference between PITG_13940 and PITG_21949 (Figure 3), and since especially the K at position 99 seems rather conserved within the functional PiAVR2/PEXRD11 members, these amino acids may play an important role in PiAVR2 effector activity. Further studies, regarding protein stability, protein-protein interactions by coimmunoprecipitation and protein localisation in the host cell (Schornack et al. 2009a) will provide clues to unravel the mechanism of this specificity.

The identification of a NES motif on all effectors with PiAVR2 activity provides new insights in effector biology. We cannot yet conclude that the NES motif is required to trigger a programmed cell death in the presence of R2 homologues, however, we postulate that it has an important function in avirulence activity, for example by targeting proteins in the cytoplasm of host cells from *Solanum*. We hypothesize that R2 homologues - PiAVR2/PEXRD11 effector family recognition is due to the constrained function of PITG_13940, PITG_21949, PITG_21645, PiAVR2 and PEXRD11, which are cytoplasmatically localized *in planta* due to their NES motif, and we postulate that PITG_05121 escape of recognition is either due to its secondary structure or instability *in*

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planta. The five functional PiAVR2/PEXRD11 variants are then exposed to the R2 proteins that belong to the cytoplasmic resistance gene family, and onset of defence triggering can occur. Protein stability studies and future comparisons with other *Avr* genes carrying RXLR motif could restrain the quest of new *Avr* genes of *P. infestans* to the RXLR effectors expressed at early stages of infection and possessing a NES motif.

Our findings provide new support for the co-evolutionary R2-PiAVR2/PEXRD11 arms-race model in the potato/*P. infestans* pathosystem. The wild *Solanum* species *S. edinense*, and *S. bjertingii* contain a mix of R2 homologues, with two and four functional variants in their genome respectively (Chapter 3), that are or not able to detect PITG_13940. Perhaps, we are facing an illustration of diversifying selection in *Solanum* evolution. In addition to adding copy numbers of an *R* gene family to increase the transcript level (Bradeen et al. 2009; Kramer et al. 2009) wild *Solanum* species keep different copies with broader recognition with an effector family to optimise the resistance to late blight.

In conclusion, our study reveals a gain of function for the six R2 homologues, Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2, and Rpi-hjt1.3, which are able to detect PITG_13940, while R2, R2-like, Rpi-abpt and Rpi-blb3 can not. Therefore it would be of interest to study the expression level of PITG_13940 in *P. infestans* isolates from potato growing areas and Mexico. Interestingly the four R2 homologues used in potato breeding programs in the last 60 years (Lokossou et al. 2009; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c) are those which do not detect the PITG_13940 effector of the PEXRD11 family. Specific functional characterisation of *R2* homologues using *PiAvr2/PexRD11* effector family has brought six new *R2* homologues variants with broader resistance spectrum, which provide more diversity and specificity for potato breeding.

Material and Methods

Microbial Strains, Plants, and Culture Conditions

Escherichia coli DH5α and DH10B and *Agrobacterium tumefaciens* strain GV3101 and AGL1 (Hellens et al. 2000; Lazo et al. 1991) were routinely grown in Luria-Bertani (LB) media (Sambrook and Russell 2001) with appropriate antibiotics at 37 and 28 °C, respectively. All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook and Russell 2001). *A. tumefaciens* strain AGL1 (Lazo et al. 1991) was transformed with individual binary vectors in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000). *Nicotiana benthamiana* plants were grown and maintained at 22 to 25 °C in controlled greenhouse under 16/8-h light-dark photoperiod.

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Gene mining and cloning of *PiAvr2/PexRD11* effector family

The *PiAvr2/PexRD11* family genes were mined from the *Phytophthora infestans* T30-4 genome annotation (Haas et al. 2009) available through the Broad Institute website at http://www.broad.mit.edu/annotation/genome/phytophthora_infestans. BLASTp search was run with the C-terminal of PiAVR2 (a.a. 66 to 116) and PEXRD11 (a.a. 64 to 114). To perform Blast searches we used as criteria an E-value cutoff at 10 using a BLOSUM62 matrix, no filter, and an ungapped alignment. To the DNA fragments of the C-terminal domain a sequence corresponding to the FLAG tag "MDYKDDDDKVKLLENS" was added on the N-terminus, in respect of future protein stability studies. Constructs were synthesized using GenScript USA Inc. Generated DNA fragments were inserted into the PacI and NotI cloning sites of Tobacco mosaic virus binary vector pJL-TRBO (Lindbo 2007). All R2 variants used came from earlier studies (Lokossou et al. 2009) and constructs were in the pKGW-MG vector (kindly provided by Dr. Andreas Untergasser, Laboratory of Molecular Biology, Wageningen, The Netherlands) in between *Rpi-blb3* regulatory elements. $Avr3a^{KI}$, and *R3a* used as positive controls were inserted in pGRAB plasmid (kindly provided by Dr. Sean Chapman, SCRI, Dundee, UK) under control of the 35S promoter and terminator.

Phylogenetic analysis of *PiAvr2/PexRD11* effector family

Phylogenetic analyses of the PiAVR2/PEXRD11 variants from *P. infestans* genome T30-4 was conducted using the Neighbour-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). Bootstrap values greater than 60% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by MEGA. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences (Tamura et al. 2007). Percentages of similarity in Table 2 were calculated using using DNASTAR Lasergene v8. ClustalW analyses were performed on the EMBL-EBI website: http://www.ebi.ac.uk/Tools/ using ClustalW and protein on the CBS (Center for Biological Sequence Analysis) website: http://www.cbs.dtu.dk/ using the NetNES 1.1 webserver predicts leucine-rich nuclear export signals (NES) in eukaryotic proteins using a combination of neural networks and Hidden Markov models.

Transient expression in planta

Agroinfiltration (*A. tumefaciens* infiltration) experiments were performed on four- to five-weeks old *N. benthamiana* plants using previously described methods (Champouret et al. 2009). The cells were collected by centrifugation (2,500 r.p.m., 15 min, and 16°C). *A. tumefaciens* strains carrying the respective constructs were mixed in a 1:1 ratio in inducing media (10 mM MgCl2, 10 mM MES, pH 5.6, and 200 mM acetosyringon), and then incubated at room temperature in the dark for 2 to 3 hours before infiltration. *A. tumefaciens* solutions were infiltrated at an OD600 of 0.2. All clones were inoculated on six leaves

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divided over two plants and two repeat experiments were made. Symptoms were monitored at three to six days after infiltration (dai).

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CHAPTER 5

Functional allele-mining with *Avr3a* reveals active *R3a* in *S. stoloniferum* and hints at two centres of *R3a* evolution in Mexico and Argentina

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Functional allele-mining with *Avr3a* reveals active *R3a* in *S. stoloniferum* and hints at two centres of *R3a* evolution in Mexico and Argentina

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Abstract

Functional allele mining with effectors facilitates the discovery of novel active resistance gene homologues (*RGH*) and sheds light on the evolution of resistance (*R*) genes. *R3a* from *Solanum demissum* is known to interact with the avirulence gene $Avr3a^{KI}$ of *P. infestans*. Here we used the two known allelic variants $Avr3a^{KI}$, and $Avr3a^{EM}$, and the paralogue *Pex147-3* to probe 177 *Solanum* genotypes representing the broad *Petota* genepool, for *R3aGH*. We identified 73 genotypes responding with different patterns of responses to one or more AVR3a variants. Further functional studies by agroinfiltration and disease test using specific *P. infestans* isolates, led to nine *Solanum* genotypes containing putative functional *R3aGH*. All these plants were originating from two different centres of diversity, Mexico and Argentina. Allele mining on the Mexican *S. stoloniferum* 554-2 revealed 29 independent *R3aGH* sequences. Ten *R3aGH* were complete, and 19 appeared to be pseudo genes. Sequence analysis revealed multiple recombination sites that suggest high rates of evolution. One R3aGH, *Rpi-sto2*, triggered cell death when coinfiltrated with $AVR3a^{KI}$ on *N. benthamiana*, and therefore, is a functional *R3a* against *P. infestans* isolates. Monitoring the genetic diversity of *Avr3a* in *P. infestans* isolates revealed an over-representation of the virulence allele $Avr3a^{EM}$, and a new allele, $Avr3a^{KI2}$ was revealed. This study shows that *R3aGH* are under an evolutionary pressure and potentially co-evolved under pressure of two geographically distinct populations of *P. infestans*. In addition, we offer to potato breeder, new *R3aGHs* that can be used in future breeding programs or in molecular engineering to develop *R* genes with broader spectrum of effector recognition.

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Introduction

Over the last century, potato breeders have introduced at least 11 late blight resistance (*R*) genes, designated *R1-R11*, from the Mexican hexaploid species *Solanum demissum* (Black et al. 1953; Malcolmson and Black 1966). In the last decade, multiple studies on other wild *Solanum* species has resulted in the localisation or cloning of 19 new Resistance genes against *Phytophthora infestans* (*Rpi*-genes) (Foster et al. 2009; Hein et al. 2009; Hein et al. 2007; Jacobs et al. 2010; Kuhl et al. 2001; Lokossou et al. 2009; Pel et al. 2009; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Song et al. 2003; Tan et al. 2008; van der Vossen et al. 2003; van der Vossen et al. 2005; Villamon et al. 2005; Vleeshouwers et al. 2008; Wang et al. 2008). The interaction between potato and *P. infestans* follows the gene-for-gene model (Flor 1971). In this model, resistance is dependent on the presence of a gene in the pathogen that makes the pathogen avirulent, but only with the host genotype possessing the corresponding *R* gene. This gene from the pathogen is called avirulence gene (*Avr*). In the presence of a corresponding R protein, an Avr protein triggers a localized programmed cell death called the hypersensitive response (HR) culminating in disease resistance. One of the best studied *R* genes thus far is *R3a* (Huang et al. 2005), which occurs at the potato *R3* locus on Linkage Group (LG) XI, (Huang et al. 2004). Comparative genomic studies of the complex *R3* locus with the corresponding *I2* locus in tomato, harbouring major genes encoding resistance to the fungus *Fusarium oxysporum* (Ori et al. 1997; Simons et al. 1998), enabled the isolation of the cytoplasmic coiled-coil nucleotide binding site leucine-rich repeat (CC-NBS-LRR) protein *R3a* (Huang et al. 2004). *R3a* is located in a Major Late Blight (MLB) locus, where *R3b*, *R5 – R11* were also mapped, and is thought to be a hot spot of *Rpi* genes (Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004; Pel et al. 2009). This locus is highly extended in *S. demissum* and harbours up to 45 type I *R3a* homologs per haplotype (Friedman and Baker 2007). Type I *R* genes show high rates of sequence exchange and correspondingly may have high homology between paralogs and high haplotypic diversity (variable gene copy number, disruption of synteny) (Kuang et al. 2005).

Recent studies have demonstrated that *P. infestans* is predicted to secrete hundreds of effector proteins that target two distinct sites in the host plant; the extracellular space with the apoplastic effectors and intracellular compartments with the cytoplasmic effectors (Bhattacharjee et al. 2006; Dou et al. 2008; Grouffaud et al. 2008; Haas et al. 2009; Kamoun 2006; Whisson et al. 2007). Oomycete cytoplasmic effectors are characterized by their N-terminal signal peptides followed by conserved motifs, RXLR, LXLFLAK, etc. (Birch et al. 2008; Birch et al. 2006; Kamoun 2006; Morgan and Kamoun 2007; Tyler 2009; Whisson et al. 2007). One of the best-studied oomycete RXLR effectors is *P. infestans Avr3a* (Armstrong et al. 2005). In early studies on a set of *P. infestans* isolates from all around the world, only two allelic variants of AVR3a were identified, named AVR3a^{KI} and AVR3a^{EM}, which differ by their amino acid (a.a.) in positions 80 and 103, respectively. In addition, two paralogues

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called PEX147-3 and PEX147-2 were identified at the *Avr3a* locus (Armstrong et al. 2005). Among these AVR3a variants, only AVR3a^{KI} has been proven to have an avirulence activity in the presence of R3a (Armstrong et al. 2005). Structure-function studies by Bos et al. (2006) have demonstrated that the C-terminal half of AVR3a^{KI} is sufficient to trigger R3a-mediated hypersensitivity. In addition to its avirulence activity, a virulence function of AVR3a^{KI} was determined, namely to suppress the cell death induced by INF1 elicitin, an apoplastic protein of *P. infestans* with features of pathogen-associated molecular patterns (PAMPs) (Bos et al. 2006; Bos et al. 2009). Deletion of the C-terminal tyrosine of AVR3a^{KI} at position 147 abolishes its ability to suppress INF1-mediated cell death whilst not affecting R3a recognition (Bos et al. 2009). Recently, the CMPG1 protein, an ubiquitin E3 ligase required for INF1-mediated cell death (Gonzalez-Lamothe et al. 2006), has been identified as one of the interactors with both AVR3a^{KI} and AVR3a^{EM} (Birch et al. 2009). Since a virulence function seems concrete for both AVR3a variants, whereas avirulence is only reserved for AVR3a^{KI}, identification of an *R* gene responding to AVR3a^{EM} became an important challenge for potato breeders. Stacking such R3a^{EM} with R3a is expected to confer a broad-spectrum resistance to different *P. infestans* isolates.

Solanum section *Petota* species occur in the Andes of South America and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). *Solanum* species sometimes easily hybridize with each other (Hawkes 1990; Jacobs et al. 2008; Spooner and Hijmans 2001), and therefore diploid, triploid, tetraploid, pentaploid and hexaploid species exist. Polyploid species can contain genes with high homology to genes in one of its progenitors, as for example found for the diploid *S. bulbocastanum* carrying *Rpi-blb1* and the polyploid *S. stoloniferum* containing the functional homologue *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008; Wang et al. 2008).

For *P. infestans*, also two geographic centres of diversity have been described. First, the Toluca Valley in Mexico, where a sexual population with both the A1 and A2 mating types is present in similar frequencies and a high degree of molecular genetic diversity occurs (Flier et al. 2003; Fry et al. 1992; Goodwin and Fry 1994; Grünwald and Flier 2005). Second, South America based on endemic coexistence of distinct lineages as well as on mitochondrial and nuclear gene genealogies studies (Gomez-Alpizar et al. 2007). These studies suggest that endemic *P. infestans* isolates may have adapted to different host species prevalent in either centre. As a consequence, *R* loci that co-evolved with *P. infestans* in South American *Solanum* species are not necessarily expected to display functional similarity to those from the Central American species, and vice versa. Recently, we could determine that *R2* was functionally restricted to Mexican *Solanum* species (Chapter 3). From an evolutionary standpoint it would be of great interest to identify functional homologues which are derived from the two different centres of diversity.

In this study we decided to screen wild *Solanum* species from North, Central and South America and identified nine genotypes with putative functional *R3a* homologues (*R3aGH*)

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originating from South (two *S. microdontum*) as well as Central America (three *S. demissum*, one *S. stoloniferum*, one *S. cardiophyllum* and one *S. ehrenbergii*). The last genotype identified is one *S. semidemissum* from unknown origin. This is the first recorded opportunity to study an *R* gene that evolved in both main centres of origin of *Solanum* species. Allele mining and sequence analyses on one selected *S. stoloniferum* genotype from Mexico revealed a great diversity of type I *R3aGH* and confirmed the presence of a functional *R3a* gene in this species.

Results

R3a/AVR3a-mediated cell death differ in agro-infection or -infiltration

To assess the spectrum and intensity level of R3a responses to the *Phytophthora infestans* effector AVR3a and its closest paralogue *Pex147-3*, we performed a coinfiltration using an *A. tumefaciens* strain containing *R3a* under its native expression elements, with *A. tumefaciens* strains containing *Avr3a*^{KI}, *Avr3a*^{EM} and *Pex147-3* independently in *Nicotiana benthamiana* leaves. After five days, a confluent cell death was observed in leaf panels that were co-infiltrated with AVR3a^{KI} but also with the paralogue PEX147-3 (Figure 1A). Also AVR3a^{EM} induced some necroses in one out of four co-infiltrated sites, but not as confluent as with AVR3a^{KI}, which is all similar to observations by Bos et al. (2006). Based on the agro-infiltration assays, we conclude that R3a quickly induces cell death at high frequencies when co-expressed with AVR3a^{KI} as well as Pex147-3, and interpret that as a strong or real HR whereas less frequent and less confluent necrosis of AVR3a^{EM} was interpreted as weak or back ground response.

Agroinfection, based on Potato Virus X system (PVX) is another *in planta* expression system which is generally more sensitive and can be used in a slightly higher throughput fashion. PVX agroinfection on *Solanum tuberosum* cultivar Desiree and *N. benthamiana* plants transformed with *R3a* expressed under native elements and wild type control plants with the two different forms of AVR and two controls (respectively pGR106::AVR3a^{KI}, pGR106::AVR3a^{EM}, pGR106::CRN2 and pGR106::empty). At 14 days post inoculation no HR was visible with the pGR106::empty and with pGR106::CRN2 HR was induced with all four genotypes, indicating that the experiment was successful (Torto et al. 2003). No visible HR was observed on *R3a* plants with pGR106::AVR3a^{KI}, but cell death induction was evident with pGR106::AVR3a^{EM} on the same plants (Figure 1B). These results are in line with the observation of Extreme Resistance (ER) induced with the Chilean *S. tuberosum* resistance protein Rx (Ross 1986) which recognizes PVX. Extreme resistance is seen with a number of *R* genes conferring resistance to viruses; it is manifested as a complete lack of both macroscopic and microscopic HR lesions and is accompanied by the absence of detectable virus accumulation (Bendahmane et al. 2002; Kang et al. 2005; Kohm et al. 1993). We

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interpreted the absence of cell death from pGR106::AVR3a^{KI} as ER in *R3a* plants. In contrast, the generally weak HR described with AVR3a^{EM} on the *R3a* plants was now observed as a localized cell death, a typical HR, and we argue that this can happen due to extremely high expression levels inherent to the virus system used. We conclude that including *Pex147-3* paralogue and *Avr3a^{EM}* to *AVR3a^{KI}* would increases the chance to detect a functional *R3aGH* when using a PVX agroinfection with *Avr3a.*

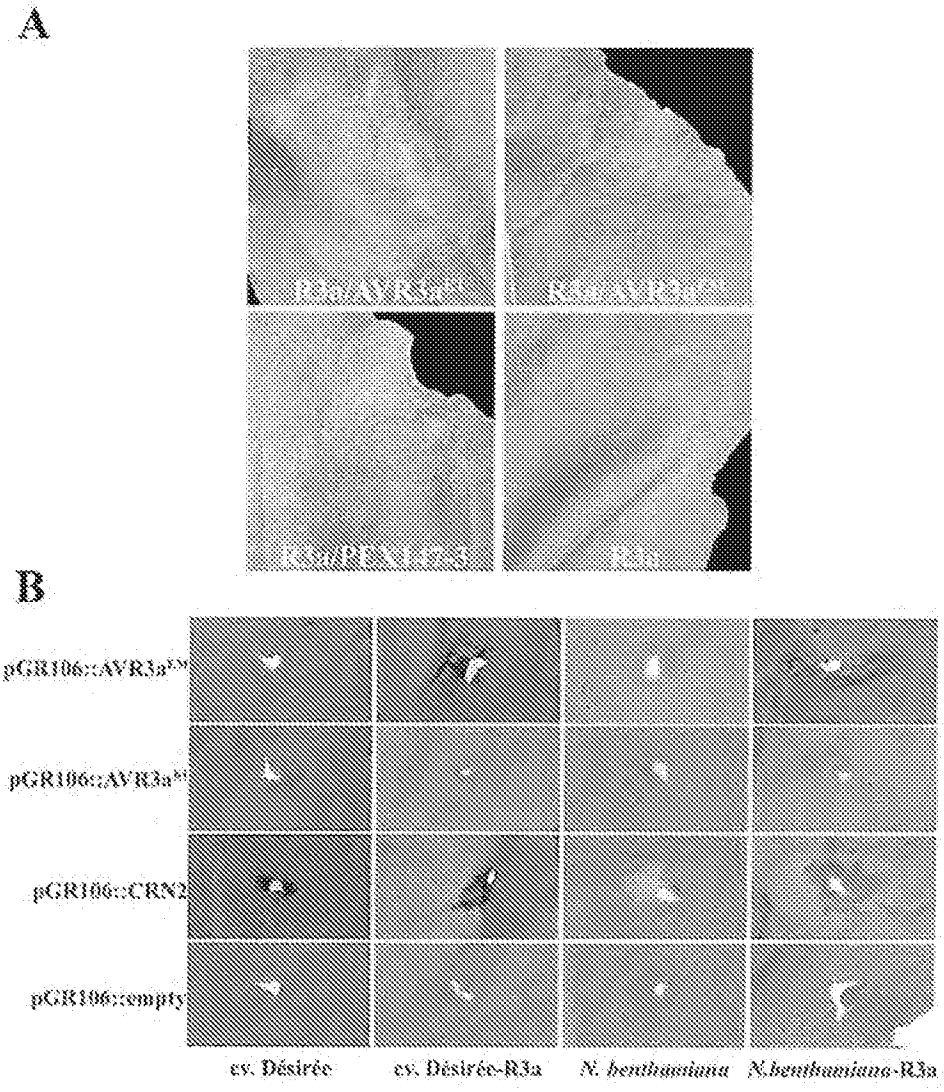


Figure 1. AVR3a triggers Hypersensitive Response or Extreme Resistance with R3a.
A) Leaves of *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain COR308 carrying pBINplus::R3a alone or mixed with *A. tumefaciens* GV3101 carrying pGR106::AVR3a^{KI}, pGR106::AVR3a^{EM} or pGR106::PEX147-3. Photographs of symptoms were taken five days post infiltration. These are a representative infiltrated spots on leaves from multiple assays and experiments. B) Leaves of *S. tuberosum* cultivar Désirée, *N. benthamiana* and R3a-transformants were wound-inoculated with *A. tumefaciens* strainsGV3101 carrying pGR106::AVR3a^{EM}, pGR106::AVR3a^{KI}, and pGR106::CRN2 and pGR106::empty vector as positive and negative controls, respectively. No visible HR is observed with pGR106::AVR3a^{KI} on R3a-transformants due to ER responses. Local cell death to pGR106::AVR3a^{EM} was observed in both R3a-transformants plants but not on *S. tuberosum* cultivar Désirée and *N. benthamiana.*

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Table 1. Functional allele-mining in *Solanum* for response to AVR3a^{KI}, AVR3a^{EM} and PEX147-3.

Series [a]	Country of origin	Solanum species	CBSG genotype code	Effectors			Controls		Group
				AVR3a^{KI}	AVR3a^{EM}	PEX147-3	R3R106 neg	CRN2 pos	
		S. tuberosum clone	SH83-92-488	+	+	+	-	+	
	Argentina	*S. acaule*	881-2	+	+	+	-	+	
	Bolivia	*S. acaule*	881-2	+	+	+	-	+	
	Mexico	*S. demissum*	343-1	+	+	+	-	+	
	Mexico	*S. acaule acroglossa*	889-4	+	+	+	-	+	
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [b]	298-2	+	+	+	-	+	
	Mexico [b]	*S. demissum* [b]	354-1	+	+	+	-	+	
	Mexico [b]	*S. demissum* [b]	364-2	+	+	+	-	+	
	Peru	*S. acaule ponce*	441-4	+	+	+	-	+	
	Peru	*S. acaule*	813-5	+	+	+	-	+	
	Unknown	*S. semidemissum*	295-1	+	+	+	-	+	
	Peru	*S. dolichocremastrum*	148-1	+	+	+	-	+	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. iopetalum*	172-4	+	+	+	-	+	
Cultivated Tuberosa and Tuberosa from Peru	Peru	*S. ocellistipetalum*	135-1	+	+	+	-	+	
Demissa Conicibaccata	Colombia	*S. mocagoanum*	725-1	+	+	+	-	+	
	Mexico	*S. schenckii*	283-2	+	+	+	-	+	
Longipedicellata	United States	*S. fendleri*	596-2	+	+	+	-	+	1
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. jesteri*	358-4	+	+	+	-	+	
	Argentina	*S. microdontum*	715-1	+	+	+	-	+	
	Argentina	*S. microdontum*	360-1	+	+	+	-	+	
	Argentina [b]	*S. tarijense* [c]	280-4	+	+	+	-	+	
	Bolivia	*Unknown species* [c]	691-1	+	+	+	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. spersipilum*	383-3	+	+	+	-	+	
	Bolivia	*S. spersipilum*	383-4	+	+	+	-	+	
	Peru	*S. brace-papa*	110-4	+	+	+	-	+	
	Peru	*S. brace-papa*	109-1	+	+	+	-	+	
	Peru	*S. raphanifolium*	793-5	+	+	+	-	+	
	Argentina	*S. venturii*	358-2	+	+	+	-	+	
Tuberosa Megistacroloba	Argentina	*S. oleadae*	367-1	+	+	+	-	+	
	Argentina	*S. oleadae*	369-8	+	+	+	-	+	
	Peru	*Unknown species* [c]	253-1	+	+	+	-	+	
		S. tuberosum clone	Florida and Ace	+	+	-	-	+	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	486-1	+	+	-	-	+	2
		S. tuberosum clone	Mis-R3	+	-	+	-	+	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-1	+	-	+	-	+	3
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. huancabambense*	354-1	-	+	+	-	+	
Longipedicellata	Mexico [b]	*S. stoloniferum* [c]	554-2	-	+	+	-	+	
	Unknown	*Unknown species* [c]	287-2	-	+	+	-	+	
	Guatemala	*S. clarum*	133-1	-	+	+	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Guatemala	*S. bulbocastanum pantlum*	120-2	-	+	+	-	+	4
	Mexico	*S. ehrenbergii*	153-3	-	+	+	-	+	
	Mexico	*S. michoacanum*	279-1	-	+	+	-	+	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Unknown	*S. neorossii*	281-2	-	+	+	-	+	
Tuberosa Megistacroloba	Argentina	*S. oleadae*	368-2	-	+	-	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. cardiophyllum*	541-2	-	+	-	-	+	5
S. verrucosum	Mexico	*S. brachycarpum*	804-8	-	+	-	-	+	
Acaulia *S. demissum*	Bolivia	*S. acaule*	316-4	-	-	+	-	+	
	Peru	*S. albicans*	461-3	-	-	+	-	+	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. huancabambense*	354-2	-	-	+	-	+	
	Colombia	*S colombianum*	574-3	-	-	+	-	+	
Demissa Conicibaccata	Costa Rica	*S. longiconicum*	180-3	-	-	+	-	+	
	Ecuador	*S. tundiomense*	247-1	-	-	+	-	+	
	Venezuela	*S. subpenduratum*	223-5	-	-	+	-	+	
Longipedicellata	Mexico	*S. stoloniferum*	835-3	-	-	+	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Bolivia	*S. megadamasii*	734-2	-	-	+	-	+	
	Bolivia	*S. megadamasii*	734-1	-	-	+	-	+	
	Argentina	*Unknown species* [c]	210-5	-	-	+	-	+	
	Argentina	*S. ruiz-leali hybrid*	802-1	-	-	+	-	+	
	Argentina	*S. microdontum*	714-1	-	-	+	-	+	
	Argentina	*S. microdontum*	360-5	-	-	+	-	+	
	Argentina	*S. gourlayi vidaurrei*	626-2	-	-	+	-	+	
	Argentina [b]	*S. tarijense* [c]	280-1	-	-	+	-	+	6
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. gourlayi pachycrinchum*	616-2	-	-	+	-	+	
	Bolivia	*S sutmeli*	477-1	-	-	+	-	+	
	Bolivia	*S. cleadae*	144-3	-	-	+	-	+	
	Bolivia	*S. microdontum*	361-2	-	-	+	-	+	
	Bolivia	*S. chacoense*	544-2	-	-	+	-	+	
	Bolivia	*S. berthaultii*	481-3	-	-	+	-	+	
	Bolivia	*S. sitmeli*	477-1	-	-	+	-	+	
	Argentina	*Unknown species* [c]	194-21	-	-	+	-	+	
	Bolivia	*S. megistacrolobum*	699-1	-	-	+	-	-	
	Peru	*S. mochiquense*	186-1	-	-	+	-	+	
Tuberosa Megistacroloba	Peru	*S. medians*	183-4	-	-	+	-	+	
	Peru	*S. multidissectum*	791-22	-	-	+	-	+	
	Peru	*S. huancabambense*	171-2	-	-	+	-	+	
	Unknown	*S. commersonii maimarum*	266-2	-	-	+	-	+	

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Table 2. *R3a* functional profiling by Agroinfiltration on the 73 wild *Solanum* genotypes.

Series [a]	Country of origin	Solanum species	CBSG genotype code	Agroinfiltration				Group
				R3a/AVR3a[c]	AVR3a[b]	AVR3a[28]	R3a	
		S. tuberosum clone	Ma-R3	+++	+++	+	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*Unknown species* [c]	881-1	++	++	++	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	715-1	++	++	++	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Guatemala	*S. clarum*	133-1	++	+	+	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Mexico	*S. ehrenbergii*	153-3	++	++	+	-	
		S. tuberosum clone	Pentland Ace	+	+	+	-	I
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	289-2	+	+	+	-	
Acaulia *S. demissum*	Mexico [c]	*S. demissum* [c]	264-1	+	+	+	-	
Acaulia *S. demissum*	Unknown	*S. semidemissum*	285-1	+/-	+/-	+/-	-	
Acaulia *S. demissum*	Mexico	*S. demissum*	343-1	+/-	+/-	+/-	-	
		S. tuberosum clone	SH83-92-488	+++	+++		-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Mexico	*S. cardiophyllum*	541-2	+++	+++		-	II
Longipedicellata	Mexico [b]	*S. stoloniferum* [c]	564-2	+++	+++		-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Bolivia	*S. neocardenasii*	734-2	+++	-	-	-	
Demissa Conicibaccata	Colombia	*S. moscopanum*	709-1	+++	-	-	-	
Demissa Conicibaccata	Ecuador	*S. lindelomense*	247-1	+++	-	-	-	
Demissa Conicibaccata	Mexico	*S. schenckii*	393-2	+++	-	-	-	
Circaeifolia Piurana Megistacrolobia Tuberosa	Peru	*S. dolichocremastrum*	148-1	+++	-	-	-	
Acaulia *S. demissum*	Peru	*S. acaule punae*	441-4	+++	-	-	-	
Tuberosa Megistacrolobia	Argentina	*S. venturii*	250-2	++	-	-	-	
Tuberosa Megistacrolobia	Argentina	*S. okadae*	356-2	++	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. goudayi pachytrichum*	516-2	++	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Guatemala	*S. bulbocastanum pertile*	130-2	++	-	-	-	
Circaeifolia Piurana Megistacrolobia Tuberosa	Peru	*S. limbo*	172-4	++	-	-	-	
Tuberosa Megistacrolobia	Peru	*Unknown species* [c]	253-1	++	-	-	-	
Circaeifolia Piurana Megistacrolobia Tuberosa	Peru	*S. huancabambense*	354-1	++	-	-	-	
Acaulia *S. demissum*	Peru	*S. albicans*	461-3	++	-	-	-	III
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*Unknown species* [c]	210-2	+	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Bolivia	*S. neocardenasii*	734-1	+	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S avilesii*	477-1	+	-	-	-	
Demissa Conicibaccata	Colombia	*S.colombianum*	574-3	+	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Mexico	*S. iopetal*	358-4	+	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. brace-pepa*	116-4	+	-	-	-	
Tuberosa Megistacrolobia	Peru	*S. mochiquense*	186-1	+	-	-	-	
Circaeifolia Piurana Megistacrolobia Tuberosa	Peru	*S. huancabambense*	354-2	+	-	-	-	
Longipedicellata	United States	*S. fendleri*	596-2	+	-	-	-	
Longipedicellata	Unknown	*Unknown species* [c]	287-2	+	-	-	-	
Tuberosa Megistacrolobia	Unknown	*S. commersonii malmeanum*	266-2	+	-	-	-	
Demissa Conicibaccata	Venezuela	*S. subpandurium*	233-3	+	-	-	-	
Acaulia *S. demissum*	Argentina	*S. acaule*	351-2	-	-	-	-	
Tuberosa Megistacrolobia	Argentina	*Unknown species* [c]	194-23	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. ruiz-lealii hybrid*	802-1	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. spegazzinii*	383-5	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. spegazzinii*	383-4	-	-	-	-	
Acaulia *S. demissum*	Bolivia	*S. acaule*	681-2	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. doddsii*	144-3	-	-	-	-	
Tuberosa Megistacrolobia did not clear due to polytomy	Bolivia	*S. megistacrolobum*	689-1	-	-	-	-	IV
Demissa Conicibaccata	Costa Rica	*S. longiconicum*	180-1	-	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Moreliformia	Mexico	*S. michoacanum*	279-1	-	-	-	-	
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	264-2	-	-	-	-	
Cultivated Tuberosa and Tuberosa from Peru	Peru	*S. ccoelestipetalum*	135-1	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. brace-pepa*	109-1	-	-	-	-	
Tuberosa Megistacrolobia	Peru	*S. medians*	183-4	-	-	-	-	
Tuberosa Megistacrolobia	Peru	*S. multidissectum*	731-22	-	-	-	-	
Tuberosa Megistacrolobia	Argentina	*S. okadae*	357-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa Megistacrolobia	Argentina	*S. okadae*	356-6	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	360-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	360-6	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. goudayi oleaunti*	526-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	280-4	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	380-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	486-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	BOLIVIA	*S. microdontum*	361-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. chacoense*	548-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	481-3	Agro.	Agro.	Agro.	Agro.	V
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. arnezii*	471-1	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Bolivia	*S. acaule*	318-4	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Mexico	*S. acaule semidens*	668-4	Agro.	Agro.	Agro.	Agro.	
*S. vernei*cosum	Mexico	*S. brachycarpum*	504-6	Agro.	Agro.	Agro.	Agro.	
Longipedicellata	Mexico	*S. stoloniferum*	836-3	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. raphanifolium*	783-5	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Peru	*S. acaule*	513-5	Agro.	Agro.	Agro.	Agro.	
Tuberosa Megistacrolobia	Peru	*S. leptophyes*	171-3	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Unknown	*S. sanctae*	281-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-3	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-1	+	+	+	+	

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Table 1. (page 84) **Functional allele-mining in *Solanum* for response to AVR3a^{KI}, AVR3a^{EM} and PEX147-3.**
73 *Solanum* genotypes belonging to various phylogenetic series (Jacobs et al. 2008) and from different countries, and three genotypes containing *R3a* (SH83-92-488, Pentland Ace, Ma-R3), were tested by PVX agroinfection for response to *A. tumefaciens* GV3101 containing, pGR106::AVR3a^{KI}, pGR106::AVR3a^{EM}, pGR106::PEX147-3, pGR106::empty vector and pGR106::CRN2. At least 16 replicates were inoculated, and (+) or (-) correspond to necrotic responses occurring or not on the inoculated sites. Leaves were monitored for developing symptoms during 16 days. Group I to VI were defined based on their pattern of response to each of the three effectors tested.
[a] Series according Jacobs et al. (2008)
[b] Deduced from Hawkes (1990)
[c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

Table 2. (page 85) ***R3a* functional profiling by Agroinfiltration on the 73 wild *Solanum* genotypes.**
73 *Solanum* genotypes belonging to various phylogenetic series selected from the agroinfection screen, and three genotypes containing *R3a* (SH83-92-488, Pentland Ace, Ma-R3), were tested by agroinfiltration for response to pGRAB::AVR3a^{KI}, and pGRAB::AVR3a^{EM}. The transient co-infiltration, used as positive control, of *A. tumefaciens* strains carrying pGRAB::R3a and pGRAB:Avr3a^{KI} were performed. At least 2 leaves per plant and 3 plants per genotypes were tested. Scoring is represented by the following symbols, (-) no response, (+/-) small response visible on 15-30% of the infiltrated spots, (+) small, (++) clear, (+++) very clear responses on 100% of the infiltrated spots, (Agro.) background responses to *A. tumefaciens*. (i) inconclusive data.
[a] Series according Jacobs et al. (2008), [b] Deduced from Hawkes (1990), [c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

Specific responses to PVX-AVR3a detected in various wild *Solanum* species

To identify wild *Solanum* species containing functional *R3a* homologues, we initiated a functional allele-mining approach with AVR3a^{KI}, AVR3a^{EM} and PEX147-3. The pGR106::empty and pGR106::CRN2 were included as negative and positive controls, respectively. As positive control on the plant side, *S. tuberosum* clone Pentland Ace, SH83-92-488, and Mastenbroek R3 (Ma-R3) were included, since they contain a functional *R3a* (Armstrong et al. 2005; Huang et al. 2005; Huang et al. 2004). We selected a phylogenetically diverse set of 177 *Solanum* genotypes originating from North, Central to South America (Jacobs et al. 2008). In total, 73 wild *Solanum* genotypes showed a response to one of the three effectors, and six different patterns of responses (group 1-6) were observed (Table 1). Group 1 contains 30 wild *Solanum* species and the positive control SH83-92-488, and all these plants display an HR upon agroinfection with pGR106::AVR3a^{KI}, ::AVR3a^{EM} and ::PEX147-3. The second group consists of *S. berthaultii* 488-1 and Pentland Ace, which both show specific cell death to pGR106::AVR3a^{KI} and ::AVR3a^{EM} but not to pGR106::PEX147-3. Group 3 consists of *S. microdontum* 714-1 and Ma-R3, which both show specific response to AVR3a^{KI} and PEX147-3 but not to AVR3a^{EM}. Since our three control plants arise in group 1-3, the identified 32 wild *Solanum* genotypes species might contain a functional R3aGH. Group 4, 5, and 6 consists of eight, three or 30 genotypes exhibiting responses to both AVR3a^{EM} and PEX147-3, AVR3a^{EM} only, or PEX147-3 only, respectively, but not to pGR106::AVR3a^{KI}. These last 41 genotypes may either contain

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R3aGH that caused ER to pGR106::AVR3a^{KI}, or they may contain a specific functional *R* gene interacting with *Avr3*^{EM}.

Response to AVR3a confirmed by agroinfiltration in 13 *Solanum* genotypes.

To validate the PVX agroinfection screen we performed an agroinfiltration on the 73 genotypes identified with pGRAB::AVR3a^{KI} and pGRAB::AVR3a^{EM}, and included SH83-92-488, Pentland Ace, Ma-R3 genotypes as positive controls. On each genotype, the R3a/AVR3a^{KI} co-infiltration was included as a positive control. Five patterns of responses were observed, represented by group I - V (Table 2). Group I contains eight wild *Solanum* genotypes, Pentland Ace and Ma-R3, which trigger HR upon infiltration with pGRAB::AVR3a^{KI} and pGRAB::AVR3a^{EM} at different levels of intensity. Group II, consists of two wild *Solanum* genotypes and SH83-92-488, show strong response to pGRAB::AVR3a^{KI} only. Since our three *R3a*-genotypes are in these first two groups, we postulate that the 12 *Solanum* genotypes in group I and II either contain an *R3aGH* responding to one or both allelic versions of *Avr3a*, or a second *R* gene interacting with *Avr3a*^{EM} in the case of genotypes from group I. Group III contains 26 genotypes all showing HR only to the positive control, and we conclude that these plants do not show any presence of functional *R3a*. Group IV and group V do not show any HR to the positive control in the first group, or show a response to the *A. tumefaciens* negative controls, respectively, indicating that the agroinfiltration on these 35 genotypes is inconclusive. *S. microdontum* 714-1 was also included in group V since results were inconclusive due to non-reproducible outcome between repeats.

Nine genotypes from Central and South America may contain a functional R3a homologue

To further study whether the responses to *AVR3a* alleles and *PEX147-3* is based on the presence of a functional *R3a* or another *R* gene, we continued with *P. infestans* isolates. First we determined the genetic variation of *Avr3a* and *PEX147-3* for a collection of 15 *P. infestans* isolates, that originated from European potato growing areas and the Central Highlands of Mexico (Champouret et al. 2009). PCR with conserved *Avr3a* primers (Table S1), on genomic DNA yielded amplicons of expected size which were cloned and a subset of 48 individual clones were sequenced. Sequence analysis revealed an over-representation of *Avr3a*^{EM} in *P. infestans* population, with ten isolates out of the 15 tested being homozygous for the virulent allele of *Avr3a* (Table 3). Also, one new variant of *Avr3a* in isolate PIC99189 and IPO-0 was discovered. This new variant has one amino acid difference compared to *Avr3a*^{KI} in position 139 where a methionine (M) is replaced by a leucine (L) and therefore we designated it *Avr3a*^{KIL} (Table 3).

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Table 3. Occurrence of *Avr3a* variants and *Pex147-3* in different isolates of *Phytophthora infestans*.

P. infestans Isolate	Country of origin	*Avr3a* and *Pex147-3* variants			
		$E^{80}M^{103}$	$K^{80}I^{103}$	$K^{80}I^{103}L^{131}$	Pex147-3
F93573	The Netherlands	x			x
PIC99177	Mexico	x			x
88069	The Netherlands	x			x
PIC99189	Mexico	x		x	x
90128	The Netherlands	x			x
EC1	Ecuador	x			x
H30P04	The Netherlands	x			x
USA618	Mexico	x			x
IPO-0	Unknown		x	x	x
IPO-C	Belgium	x	x		x
PIC99183	Mexico	x			x
NL01096	The Netherlands	x			x
VK98014	The Netherlands		x		x
IPO428-2	The Netherlands	x			x
NL00228	The Netherlands		x		x

To test specific resistance responses to the three AVR3a variants, we produced zoospore suspensions of IPO-0 (*Avr3a*^{KI} - *Avr3a*^{KIL}), PIC99189 (*Avr3a*^{EM} -*Avr3a*^{KIL}) and PIC99183 (*Avr3a*^{EM} *homozygote)* and inoculated the 73 selected genotypes from the agroinfection screen (Table 1) in addition to three *R3a* control genotypes (data not shown). All three *R3a* controls were resistant to both isolates IPO-0 and PIC99189, and susceptible to PIC99183, as expected. Overall susceptibility to all three *P. infestans* isolates was found for 41 wild *Solanum* genotypes, which were classified in group 1,2,4,5,6 (by agroinfection) and III – V (by agroinfiltration), and these plants were discarded from further analysis. Eleven more genotypes were susceptible to one of both isolates containing an *Avr3a*^{KI} form; as a result they were removed from the genotype pool studied. Twelve other genotypes were fully resistant to all three isolates; seven of them displayed no AVR3a response and showed adequate responses to the agroinfiltration controls (Table 2), and five showed responses to the *A. tumefaciens* negative controls (Table 2). In the first seven cases we expect the resistance to be due to another *R* gene, different from *R3aGH*, and did not include them for further study. In the second five cases we could not confirm R3a activity by agroinfiltration and set aside these genotypes for future studies (Table 4B). In total nine genotypes remained (Table 4A). Seven of these, i.e. *S. demissum* (dms) 299-2, *S. semidemissum* (sem) 295-1, *S. microdontum* (mcd) 714-1, *S. microdontum* (mcd) 715-1, *S. stoloniferum* (sto) 554-2, *S. cardiophyllum* (cph) 542-2 and *S. ehrenbergii* (ehr) 153-3 show an R3a-specific resistance to the *P. infestans* isolates, and similar responses to AVR3a as the R3a-genotypes, suggesting that these plants might contain functional R3a homologues. Two other genotypes dms343-1, and sem264-1 also displayed a similar pattern of responses to the *Avr3a* variants, and showed resistance even to *P. infestans* isolate PIC99183, suggesting presence of a functional *R3a* homologue that also responds to *Avr3a*^{EM} or another *R* gene responding to another effector in PIC99183.

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Table 4. Functional profiling reveals the presence of *R3a* homologues in diverse *Solanum* species from Central and South America.

Series [a]	Country of origin	*Solanum* species	Species abbreviation	CB5G genotype code	Agroinfection					Agroinfiltration				*P. infestans* isolates		
								neg.	pos.							
					AVR3aKI	AVR3aEM	Pex147-3	pGR106	Crn2	R3a/AVR3aKI	AVR2aKI	AVR3aEM	R3a	IPO-0 (Avr3a^{KI}, Avr3a^{EM})	P/C89189 (Avr3a^{KI}, Avr3a^{EM})	P/C89183 (Avr3a^{EM})
		S. tuberosum clone		SH83-92-488	+	+	+	-	+	+++	+++	-	-	R	R	S
		S. tuberosum clone		*Pentland Ace*	+	+	-	-	+	+	+	+	-	RQ	R	S
		S. tuberosum clone		Ma-R3	+	-	+	-	+	+++	+++	+	-	R	RQ	S
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	dms	298-2	+	+	+	-	+	+	+	+	-	R	R	S
Acaulia *S. demissum*	Unknown	*S. semidemissum*	sem	295-1	+	+	+	-	+	++	+/-	+/-	-	R	R	S
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	mcd	714-1	+	-	+	-	+	!	!	!	!	R	R	S
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	mcd	715-1	+	+	+	-	+	++	++	++	-	R	R	S
Longipedicellata	Mexico [b]	*S. stoloniferum* [c]	sto	554-2	-	+	+	-	+	+++	+++	-	-	R	R	S
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. cardiophyllum*	cph	541-2	-	+	-	-	+	+++	+++	-	-	R	R	S
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. ehrenbergii*	ehr	153-3	-	+	+	-	+	++	++	+	-	R	R	S
Acaulia *S. demissum*	Mexico	*S. demissum*	dms	343-1	+	+	+	-	+	!	+/-	+/-	-	R	R	R
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	dms	264-1	+	+	+	-	+	+	+	+	-	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	tar	280-4	+	+	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa Megistacroloba	Argentina	*S. okadae*	oka	367-1	+	+	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	tar	280-1	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	ber	481-3	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Longipedicellata	Mexico	*S. stoloniferum*	sto	835-3	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R

A) Solanum genotypes with confirmed *R3aGH* functional content due to the different phenotypes on the three biological assays. B) *Solanum* genotypes with *R3aGH* functional content inaccurate due to the results of agroinfiltration assays. Agroinfection and Agroinfiltration scoring are as shown in Table 1 and 2. Detached leaf assay: *Solanum* genotypes were challenged by three *P. infestans* isolates and scored for their Resistance (R), partial resistance (RQ) or susceptibility (S).

[a] Series according Jacobs et al. (2008)

[b] Deduced from Hawkes (1990)

[c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

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In summary, our functional profiling approach combined with resistance tests lead to the identification of nine diverse *Solanum* genotypes which are likely to contain a functional *R3a* homologue. These plants originate from four different phylogenetic series, i.e. Acaulia S. Demissum; Tuberosa from Bolivia, Argentina and Chile Yungasensa; Longipedicellata; and Polyadenia Pinnatisecta Bulbocastana Morelliformia; and two different main centres of origin, Mexico and Argentina.

Genetic and pathogenic studies support presence of a functional *R3a* gene in *S. stoloniferum* 22718-2

To genetically test the presence of a functional *R3a* homologue, *S. stoloniferum* 554-2 was crossed with the susceptible *S. stoloniferum* 297-1, which did not respond to $Avr3a^{KI}$ or $Avr3a^{EM}$ (Table 5A). We examined 50 individuals of the F1 population for response to *Avr3a* variants by agroinfiltration and observed a HR to $AVR3a^{KI}$ by each individual of the population. We also assayed all the individuals of the F1 progeny for segregation of resistance to two isolates of *P. infestans*, IPO-0 and 90128, which are avirulent and virulent, respectively, on R3a-plants. All individuals in the F1 population and sto554-2 were resistant to IPO-0, and all genotypes were susceptible to 90128 (Table 5B). Since no segregation in the progeny was detected, the responsible *R* gene is most likely present in a homozygous state in sto554-2, and conclusions on co-segregation for effector response and *P. infestans* resistance could not be drawn. However, the results were still in line with the hypothesis, that a functional *R3a* might be present in sto554-2.

Table 5. Reaction pattern and population study of *S. stoloniferum* 554-2 reveals the presence of a functional and homozygous *R3a* homologue in parent 554-2.

		A				B	
Solanum species	**Genotype code**	**Agroinfiltration**				***P. infestans* isolates**	
		R3a/AVR3aKI	**AVR3aKI**	**AVR3aEM**	**R3a**	**IPO-0 ($Avr3a^{KI}$ - $Avr3a^{KO}$)**	**90128 ($Avr3a^{EM}$)**
S. stoloniferum	554-2	+++	+++	-	-	R	S
S. stoloniferum	297-1	+++	-	-	-	S	S
Offspring							
Genotypes 1 to 50		+++	+++	-	-	R	S

A) Agroinfiltration: At least two leaves per plant and three plants per genotypes were tested. Scoring is represented by the following symbols, no response (-), (+++) very clear response on 100% of the infiltrated leaf panel. B) Detached leaf assay: *Solanum* genotypes were challenged by five *P. infestans* isolates and scored for their Resistance (R), or Susceptibility (S).

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We then tested whether R3a-specific resistance was associated with specific Avr3a variants in the *P. infestans* isolates. We inoculated sto554-2 with ten *P. infestans* isolates that carry diverse *Avr3a* variants in homozygous or heterozygous state (Armstrong et al. 2005). Cultivar *S. tuberosum* cv. Impala and *S. bulbocastanum* 2002 containing the broad-spectrum *Rpi-blb2* gene were susceptible and resistant respectively to all isolates tested, which indicates that the assay was reliable. Sto554-2 was specifically resistant to the four isolates containing an $Avr3a^{KI}$ or $Avr3a^{KIL}$ and susceptible to the six isolates homozygous for $Avr3a^{EM}$ (Table 6). We conclude that the resistance spectrum of sto554-2 supports the presumed presence of an *R3a* homologue.

Allele mining reveals a high diversity of *R3a* homologues in *S. stoloniferum*

To assess the diversity of *R3a* in sto554-2, we performed an allele mining approach. PCR analyses, using a combination of conserved primers for *R3a* and *R3a* paralogues (*I2GA-SH194-2, I2GA-SH23-1, I2GA-SH23-3*), on genomic DNA of genotype sto554-2 yielded amplicons of 3200 to 4000bp. *R3a* and *R3a* paralogues described by Huang et al. (2005) range from 3798 to 3984bp. In total 29 non-redundant sequences were cloned from this genotype and designated *stoR3aGH1* to 29. Sequence analysis revealed that two sequences did not have a stop codon (*stoR3aGH28* and *29*) and seventeen sequences possessed an additional early stop codon on their Coiled-coil (*stoR3aGH22* to *27*), NBS domains (*stoR3aGH15* to *21*) or first half of the LRR (*stoR3aGH11* to *14*) domains. In total ten full length sequences, from 3513 to 3984bp, with an *R* gene structure were identified (*stoR3aGH1* to *10*), with nucleotide identities from 87.2% to 99.9% compared to *R3a* and *R3a* paralogues (Table 7). StoR3aGH5 is most similar to R3a and has only four amino acids different, i.e. one in the CC domain, one in subdomain LRR18, and two in subdomain LRR21 (Figure 2). Another clone, stoR3aGH4, has only 89.2% of a.a. similarity with R3a (Table 7) and shows only three different a.a. compared to I2GA-SH23-3, a non-functional R3a paralogue described by Huang et al. (2005). Two sequences, stoR3aGH3 and stoR3aGH10 are synonymous and have only one nucleotide difference in their NBS domain (data not shown). Sequence alignment shows that recombination events have occurred in the past, i.e. stoR3aGH8 has a first half of its CC domain identical to stoR3aGH9, and then from this point to the LRR13 subdomain, stoR3aGH8 is similar to stoR3aGH5, with only one a.a. change in LRR4 subdomain. In the LRR13 subdomain a new recombination event occurs and the stoR3aGH8 got the C-terminal domain of stoR3aGH7, with only two a.a. changes in the LRR28 subdomain of R3a alignment (Figure 2). The phylogenetic relationship between the *R3a* variants was examined by constructing a Neighbour-joining (NJ) tree (Figure 3). The tree was rooted with *I2* from *Solanum lycopersicum*, the closest known homologue of *R3a* in plants (Huang et al. 2005). The phylogenetic tree shows that the *R3a* gene family is highly diverse and extended in *S. stoloniferum*.

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Table 6. Pathogenic assay supports the presumed presence of an *R3a* homologue in *S. stoloniferum* 554-2.

P. infestans isolates			Genotypes			
	Origin	*Avr3a* content	*S. tuberosum* cv. Impala	*S. stoloniferum* 554-2	*Ma-R3a*	*S. bulbocastanum* 2002 (*Rpi-blb2*)
IPO-0	The Netherlands	Heterozygote $Avr3a^{KI}$ - $Avr3a^{EM}$	S	R	R	R
PIC99189	Mexico	Heterozygote $Avr3a^{KI}$ - $Avr3a^{EM}$	S	R	R	R
Sc95.17.3.2	Scotland	Heterozygote $Avr3a^{KI}$ - $Avr3a^{EM}$*	S	R	R	R
CA-65	U.S.A.	Homozygote $Avr3a^{KI}$*	S	R	R	R
H3OPO4	The Netherlands	Homozygote $Avr3a^{EM}$	S	S	S	R
90128	The Netherlands	Homozygote $Avr3a^{EM}$	S	S	S	R
PIC99177	Mexico	Homozygote $Avr3a^{EM}$	S	S	S	R
PIC99183	Mexico	Homozygote $Avr3a^{EM}$	S	S	S	R
88133	The Netherlands	Homozygote $Avr3a^{EM}$*	S	S	S	R
Sc96.9.5.1	Scotland	Homozygote $Avr3a^{EM}$*	S	S	S	R

Detached leaf assay: *Solanum* genotypes were challenged by ten *P. infestans* isolates and scored for their Resistance (R), or Susceptibility (S).
* *Avr3a* content according to (Armstrong et al. 2005).

Table 7: Percentage similarity between the ten *StoR3aGH* genes and *R3a*.

	stoR3aGH1	stoR3aGH2	stoR3aGH3	stoR3aGH10	stoR3aGH4	stoR3aGH5	stoR3aGH6	stoR3aGH7	stoR3aGH8	stoR3aGH9	R3a	I2
stoR3aGH1	***	88.3	89	89.1	88.3	94.8	94.9	89.4	94.5	90	94.8	87.3
stoR3aGH2	12.7	***	96.6	96.6	94.9	88.4	89.4	94.6	89.3	89.9	88.4	87.7
stoR3aGH3	11.9	3.5	***	99.9	91.3	89	90.1	93.2	89.9	90.4	89.1	87.6
stoR3aGH10	11.8	3.5	0.1	***	91.4	89	90.1	93.3	90	90.4	89.1	87.7
stoR3aGH4	12.8	5.3	9.2	9.2	***	89.1	89	93	88.6	90.5	89.2	86.9
stoR3aGH5	5.4	12.6	11.9	11.9	11.8	***	98.1	89.3	96.2	92.3	99.9	88.1
stoR3aGH6	5.3	11.4	10.7	10.6	12	1.9	***	90.2	96.6	92.6	98.1	88
stoR3aGH7	11.4	5.6	7.1	7.1	7.4	11.5	10.5	***	92.8	90.5	89.4	88.2
stoR3aGH8	5.7	11.6	10.8	10.8	12.3	3.9	3.5	7.6	***	92.6	96.2	87.6
stoR3aGH9	10.7	10.9	10.3	10.2	10.2	8.1	7.8	10.2	7.9	***	92.4	87.5
R3a	5.4	12.6	11.9	11.8	11.7	0.1	1.9	11.4	3.9	8.1	***	88.2
I2	13.9	13.4	13.5	13.5	14.4	12.9	13.1	12.8	13.6	13.7	12.8	***

Nucleotide sequence distances of the ten *R3a* variants of *S. stoloniferum* 554-2 compared to *R3a* and *I2*. The percentage of similarity in the upper triangle, the percentage of divergence in the lower triangle.

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Figure 2. Protein alignment of R3a variants reveals a diverse set of R3a homologues in *S. stoloniferum* 554.2.

Figure 2. (Previous page) **Protein alignment of R3a variants reveals a diverse set of R3a homologues in *S. stoloniferum* 554-2.**
The R3a amino acid sequence is used as reference and only positional amino acids changes are noted for the other sequences. Positions of Coiled coil domain, the nucleotide binding (NB)-ARC and leucine-rich repeat are indicated by arrows. The coiled coils described by Huang et al., (2005) are underlined. The NB-ARC domain includes the Kinase 1a, Switch, Kinase 2, Kinase 3a motifs of the NBS and RNBS-C, GLPL, RNBS-D and MHDV motifs underlined in that order (Huang et al. 2005). The leucine-rich repeat domain is composed of 30 LRR repeats, in bold, with the motif xxLxLxx. New LRR repeats from new R3aGH are bold and underlined. Cross represent putative recombination site, the first cross between StoR3aGH8 and StoR3aGH5, and the second cross between StoR3aGH8 and StoR3aGH7.

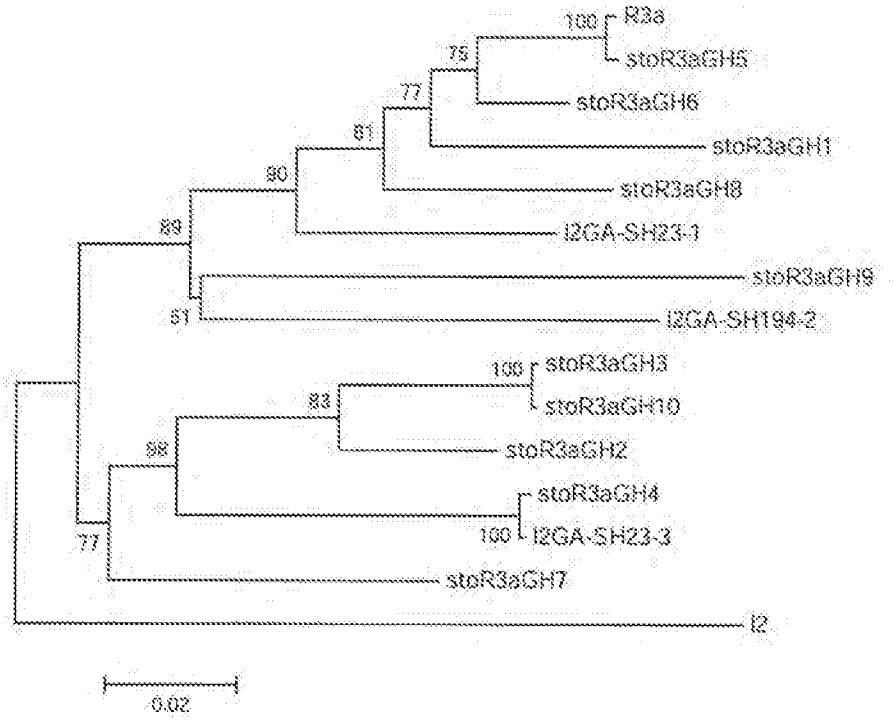


Figure 3. The R3a family is composed of highly diverse variants.
Neighbour-joining tree illustrating phylogenetic relationship of R3a variants from *S. stoloniferum* 554-2 with I2 vs. Rpi-blb1 as out group. Sequences are grouped in two clades. Bootstrap values greater than 50% (based on 1000 resample) are indicated at the nodes.

R3a homologue Rpi-sto2 from *S. stoloniferum* 554-2 is responding to AVR3a^{KI}

To evaluate the functionality of the 26 clone *R3a* homologues from sto554-2 we co-infiltrated R3a and stoR3aGH1 to 29 in *N. benthamiana* leaves with AVR3a^{KI} and AVR3a^{EM} respectively (Figure 4). StoR3aGH5 specifically induced cell death when co-expressed with AVR3a^{KI}, and slight necroses with AVR3a^{EM} like for R3a, whereas all other stoR3aGH did not (Figure 4). This shows that stoR3aGH5 is a functional *R3a*, and according to the common Rpi gene nomenclature, it was designated *Rpi-sto2*. A high degree of auto-necrosis induction was visible with three other R3a homologues, i.e. stoR3aGH4, stoR3aGH14 and

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stoR3aGH22 (Figure 4). StoR3aGH22 has an early stop codon at the end of the coiled coil domain. StoR3aGH4 has only three a.a. difference compared to I2GA-SH194-2, in LRR4, between LRR 25 and 26, and stoR3aGH14 is more diverse than any known R3a homologue. These results show that Rpi-sto2 recognizes AVR3a^{KI}, which explains the resistance of *S. stoloniferum* 554-2 to *P. infestans* isolates expressing any form of AVR3a^{KI}.

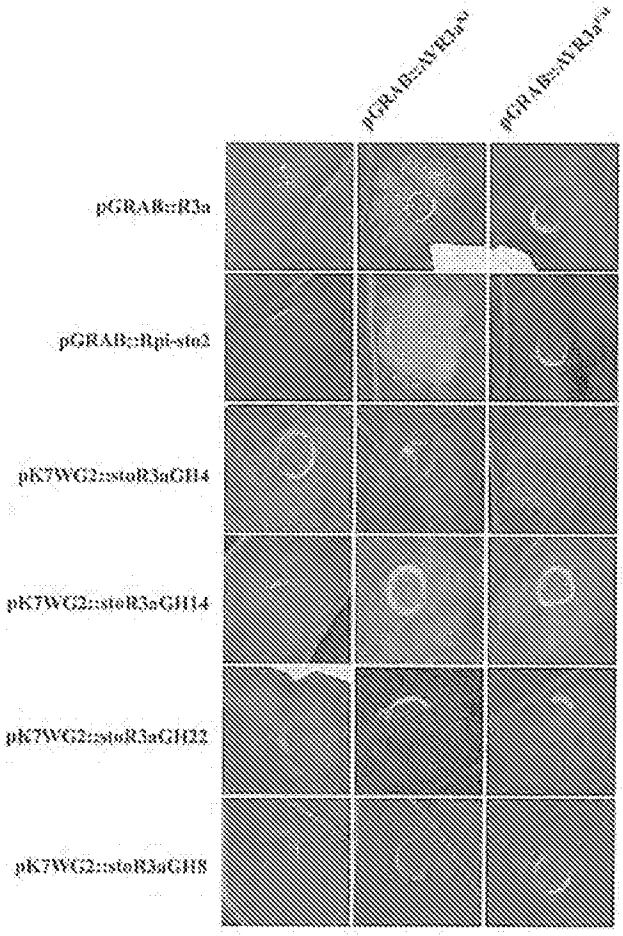


Figure 4. StoR3aGH5, but no other R3a homologue of *S. stoloniferum* 554-2, specifically recognizes AVR3a^{KI}.

Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium tumefaciens* strain AGL1 to co-express the R3a and R3a homologues of *S. stoloniferum* 554-2 with AVR3a^{KI} and AVR3a^{EM}. The R3a and StoR3aGH5 with AVR3a^{KI} combination were the only one to induce the hypersensitive response (HR) starting from three days post-infiltration (dpi). StoR3aGH4, 14 and 22 shows an auto-necrosis symptom and all other R3a homologues from *S. stoloniferum* 554-2, like StoR3aGH8, did not trigger any cell death. Photographs were taken at five dpi.

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Discussion

Identification of geographical origin of an *R* gene is valuable information for breeders, since the natural *R* gene diversity, due to its co-evolution with the pathogen, can be used to get a broader resistance. In recent studies, we determined that the *R2* late blight resistance gene was functionally restricted to *Solanum* species from Mexico (Chapter 3) and identified a centre of co-evolution between *R2* and *PiAvr2/PexRD11* effector family members (Chapter 4). In this study, we focus on *R3a*, a constitutively expressed potato gene that confers race-specific resistance to the late blight pathogen *P. infestans*. *R3a* was introgressed from the Mexican *Solanum* species, *S. demissum* (Black et al. 1953; Malcolmson and Black 1966). Previous studies have mentioned co-existence of the *R3a*-harboring species *S. demissum* with *P. infestans* in the cool and humid mountain forests in Toluca Valley of Mexico, to be a perfect condition for a tight co-evolution between *R3a* and *Avr3a* (Armstrong et al. 2005; Huang et al. 2005). These conditions, therefore, could be considered as a main driving force for tight co-evolution between *R3a* and *Avr3a*. However it was never demonstrated that other species than *S. demissum* and *Solanum* species outside Mexico could also harbour a functional *R3aGH*. Here we identified nine genotypes containing a putative functional *R3aGH*, which were originating from two different centres of diversity, Mexico and Argentina. Moreover we also identified a functional R3aGH from *S. stoloniferum*, Rpi-sto2, which interact with AVR3a^{KI}. Sequence analysis of the *R3aGH* from both centres will reveal more information of the evolution of *R3a* under two different *P. infestans* populations' pressure.

R3a originate from a Major Late Blight (MLB) locus on LG XI which is highly extended in *S. demissum* (Friedman and Baker 2007). In the tetraploid sto554-2 we identified 29 independent sequences, with some potential recombination sites like in R-stoR3aGH8, which confirm the potentially high rate of evolution in this cluster. However, our finding also suggests that the number of *R3aGH* per haplotype is much less than in *S. demissum* (Friedman and Baker 2007). This difference of *R3aGH* amplified might be linked to our approach using specific primers for *R3a* and *R3a* paralogues instead of sequenced BACs (Bacterial Artificial Chromosome) from this locus in *S. stoloniferum*. However the difference is striking and it perhaps represents a difference of evolutionary rate of the *R3a* locus between *S. demissum* and *S. stoloniferum*. In addition, among all identified *stoR3aGH* sequences, only 14 out 29 were coding for a full length *R* gene, and only one *Rpi-sto2* was functional for AVR3a activity. Since all stoR3aGH have still a high homology to R3a and its paralogues, and therefore are likely to be used for further sequence exchange in this hot-spot, this is in line with the Trench-Warfare-model (Stahl et al. 1999).

Avr3a allele-mining on the 15 *P. infestans* isolates showed that *Avr3a* contains only little diversity, and is mostly represented by *Avr3a*^{KI} and *Avr3a*^{EM}, in line with previous work (Armstrong et al. 2005). A newly detected variant *Avr3a*^{KI_L}, which thus far seems to have

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similar specificity as $Avr3a^{KI}$ because it did not evade *R3a* recognition based on disease test, appeared in the Mexican isolate PIC99189 and IPO-0, from unknown origin. Interestingly, $Avr3a^{EM}$ was found predominantly present in almost all isolates. In ten out of 15 isolates $Avr3a^{EM}$ was even the only Avr3a variant, which is in line with a generally defeated *R3a* in the potato fields (Black et al. 1953; Malcolmson and Black 1966). The abundance of $Avr3a^{EM}$ vs. $Avr3a^{KI}$ suggests that *P. infestans* isolates evolved to evade *R3a* recognition. On the other hand, wild *Solanum* species have maintained *R3a* in the population and most likely even in two different geographical regions. Therefore we hypothesise that despite the effort of the pathogen to evade $Avr3a^{KI}$ recognition, the virulence function of $AVR3a^{KI}$, such as suppression of INF1-mediated cell death through CMPG1 protein (Birch et al. 2009; Bos et al. 2009), is important enough to maintain it, and resulted in the sustainability of *R3a* in wild *Solanum* species. In conclusion, for the quest of durable resistance against late blight, it would be of interest to identify, or create by shuffling or swapping approaches, a R3a variant which responds to $AVR3a^{EM}$ at similar levels as $Avr3a^{KI}$

R gene allele mining is a powerful approach, but can also be inefficient since it does not make any distinction between genotypes containing functional or non-functional RGH. Use of gene-specific molecular markers (diagnostic marker) to identify functional *RGH* in *Solanum* still leads to diverse rates of efficiencies. For example 98% accuracy was obtained for *I2* tomato blight resistance gene (Arens et al. 2010), but only 27% for the *Rpi-blb3* potato blight resistance gene (Lokossou et al. submitted), mainly due to the high recombination events present in this *R* gene cluster (Lokossou 2010). Moreover, the number of *RGH* per genotype can depend on ploidy level, and localization in MLBs (Friedman and Baker 2007; Lokossou et al. 2009; Pel et al. 2009). Here we have combined three different techniques to gradually reduce the number of genotypes which potentially contained a functional R3aGH. PVX agroinfection is the most facile assay available for *in planta* testing of an effector on a large scale of *Solanum* genotypes (Vleeshouwers and Rietman 2009). However, interpretation of results can be difficult since many *R* genes that induce ER, including *Rx*, also induce HRs depending on the strain of the virus, host genetic background and environmental conditions (Baurès et al. 2008; Bendahmane et al. 1999; Kang et al. 2005). We anticipated on this by including other *Avr3a* variants with slightly altered levels of sensitivity, and use PVX agroinfection with Avr3a mainly as a high-trough-put pre-screening. The 73 identified genotypes, which gave responses to any of the two Avr3a alleles and/or to the paralogue Pex147-3, where than further studied by agroinfiltration, the currently best developed and most reliable method for reconstructing the R-AVR interaction in plants (Armstrong et al. 2005; Bos et al. 2006; Champouret et al. 2009; Lokossou et al. 2009), and 12 were confirmed to show response to $AVR3a^{KI}$. However, because also *A. tumefaciens* is a plant pathogen, various defence responses from the host can be initiated (Vleeshouwers and Rietman 2009), and in our case, only 54% of tested *Solanum* genotypes could reliably be tested. Another factor that contributes to the deviation of efficiency is explained by the

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absence of PEX147-3 in the agroinfiltration test. The high number of genotypes showing responses to this paralogue of *Avr3a* remains questionable, and we postulate an ancient recognition of this effector by most of the wild *Solanum* species, which would explain why almost no isolates of *P. infestans* express this effector (Haas et al. 2009; Whisson et al. 2007). In conclusion, this study suggests that the sensitive agroinfection technique in combination with agroinfiltration and disease assays provide an efficient platform for *in planta* testing of large numbers of genotypes containing *RGH* candidates of extended gene families, like *R3a* of the MLB on LG XI.

Future late blight resistance breeding can explore *RGH* with broader specificity, and deploy *R* genes that recognize the full spectrum of Avr alleles. Also molecular engineering of *R* gene could be a solution to late blight management using the *R3aGH* diversity information. Here we propose four domains to investigate for future researches. First, towards our results on R3a response to AVR3a^{EM} in transient assays, it would be of interest to transform potato cultivars with R3a under a highly constitutive promoter or with multiple copies inserted, to increase the transcription level *in planta*, and therefore, obtain broader resistance like for the enzyme resistance (*eR*) genes At1 and At2 from wild Melon (Taler et al. 2004) or with the RB/Rpi-blb1 against potato late blight (Kramer et al. 2009). A second approach would be to shuffle *R3a* to synthetically evolve this resistance gene against AVR3a^{EM}. This approach proved to be successful with Rx, which by shuffling could respond to different variants of PVX but also to a distantly related poplar mosaic virus (Farnham and Baulcombe 2006). Therefore the amplification of *R3aGH* on the remaining nine genotypes identified in our study and particularly on the Argentinean *S. microdontum*, would provide even more diversity of sequences to start with. The third opportunity is to screen additional wild *Solanum* species to find a natural genotype responding to AVR3a^{EM}. The two dms343-1 and dms264-1 as well as in the unclassified *Solanum* genotype 287-2 are the three first candidates to test for functional *R3a* homologue responding to the virulent variant *Avr3a*^{EM}. The fourth domain of investigation is based on the diversity of *R3aGH* we have uncovered. We can expect that the high diversity of *R3aGH* sequences in *S. stoloniferum* could bring recognition against other effectors than the Avr3a effector family. Therefore screening of these *R3aGH* genes against more RXLR effectors of *P. infestans* or effectors from other pathogen of potato could reveal new specificities and more understanding on R/AVR interactions and *R* gene cluster evolution.

Materials and Methods

Plant materials

The wild *Solanum* plant material used for effector screening and functional allele mining is listed in Table 1. The genotypes were retrieved from the accessions in the Centre for Genetic

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Resources, the Netherlands (CGN), and information on species, origin, collection site, GPS coordinates and other genbank code are accessible on the Sol*R*gene database (*Sol*Rgene database). SH83-92-488 (Huang et al. 2005; Huang et al. 2004), Pentland Ace (provided by Paul Birch, SCRI, Scotland, U.K.), Ma-R3 (Mastenbroek 1953) genotypes, were used as R3a control plants in agroinfection and agroinfiltration assays. *S. tuberosum* cultivar Désirée and *N. benthamiana* and there respective transformants *S. tuberosum* cultivar Désirée-R3a and *N. benthamiana*-R3a transformed with R3a under its native expression elements in pBINPlus (van Engelen et al. 1995) were used in Figure 1B. Taxonomic series information was extracted from the data in Jacobs et al., (2008) paper. The population generated for genetic studies originated from the *P. infestans* resistant *S. stoloniferum* (CBSG: 554-2) crossed with the susceptible *S. stoloniferum* (CBSG: 297-1). Plant genotypes were maintained *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18 °C. Top shoots were transferred to fresh medium, and two weeks later, rooted plantlets were transferred to the greenhouse. *Nicotiana benthamiana* plants were grown from seeds in the greenhouse and subjected to agroinfiltration experiments in climate chambers at an ambient temperature of 22–25 °C and high light intensity.

Microbial strains and growth conditions

Agrobacterium tumefaciens strains GV3101 (Hellens et al. 2000), COR 308 (Hamilton et al. 1996), and AGL1 (Lazo et al. 1991) were used in molecular cloning experiments and were routinely cultured at 28 °C in Luria–Bertani (LB) media using appropriate antibiotics (Sambrook and Russell 2001). All bacterial DNA transformations were conducted by electroporation in DH10B cells (INVITROGEN) or heat shock in DH5α cells (INVITROGEN) using the supplier recommendations.

***Phytophthora infestans* isolates and disease tests**

The *P. infestans* isolates F95573, PIC99177, 88069, PIC99189, 90128, EC1, H3OPO4, USA618, IPO-0, IPO-C, PIC99183, NL01096 and VK98014 (Champouret et al. 2009) were cultured on rye sucrose agar medium in the dark at 15 °C for 1–2 weeks (Caten and Jinks 1968). Sporulating mycelium was flooded with cold water and the sporangiospore suspension was incubated at 4 °C for 3 h. After the release of the zoospores, the inoculum was adjusted to a concentration of $5*10^4$ spores/ml. Leaves from 6-8 week old plants were detached, placed in water-saturated oasis in humid trays, and leaflets were spot-inoculated with the spore suspension. The trays were incubated in a climate chamber with a 16/8h light/dark regime at 15 °C. At six days post inoculation (dpi), the lesion sizes (LS) were determined. Sporulating lesions exceeding 15 mm were scored as susceptible (S), whereas spots displaying localized HR smaller than 5 mm at the inoculation sites were scored as resistant (R).

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PCR allele mining

All primers and plasmids used in this study are described in Table S1. To clones *Avr3a* variants from the different *P. infestans* isolates we performed PCR amplification with Phusion High-Fidelity DNA Polymerase (FINNZYMES) on genomic DNA extracted from fresh mycelium tissue of each isolate using the primers PEX147-F and PEX147-R. After 30 cycles, Super*Taq* polymerase (HT Biotechnology, Cambridge) and its buffer were added, followed by 15 min at 72 °C. The yielded amplicons of 450bp, in range for *Avr3a* homologue, were ready to be cloned into pGEM-T Easy vector (Promega). *R3aGH* variants from *S. stoloniferum* 554-2 were cloned by PCR amplification on genomic DNA using Phusion High-Fidelity DNA Polymerase (FINNZYMES). Two amplifications were made to amplify all R3a and R3a paralogues, using the conserved start primer R3a-GatBP-F2 containing the attB1 gateway, in combination with the stop primers R3a-GatBP-R2 and SH194-2-GatBP-R2 containing the attB2. All PCR fragments were purified on 1% TBE gel using Min Elute™ Gel Extraction Kit (QIAGEN). Sequencing was performed using universal M13 primers and specific primers for R3aGH (Table S1). DNA sequences were analyzed using DNASTAR Lasergene v8 and ClustalW.

Plasmid constructs

All effectors in the potato virus X (PVX) binary vectors, i.e. pGR106::empty, pGR106::*Cm2*, pGR106::*Avr3a*^{KI}, pGR106::*Avr3a*^{EM} and pGR106::*Pex147-3* (kindly supplied by Dr. S. Kamoun and Dr. J. I. B. Bos, OARDC, Wooster, USA) used in agroinfection test were without signal peptide and without RXLR motif. Agroinfiltration screens were made with *Avr3a*^{KI}, *Avr3a*^{EM} and *R3a* in pGRAB plasmid (kindly provided by Dr. S. Chapman, SCRI, Scotland, U.K.) under 35S promoter and terminator. Effectors were cloned without their signal peptide. pBINplus::*R3a* was provided by Huang et al. (2005). *Avr3a* variants amplicons from the different *P. infestans* isolates tested were cloned in pGEM®-T Easy Vector Systems (Promega) following the supplier recommendations. The purified *R3aGH* amplicons from *S. stoloniferum* 554-2 were used in a BP reaction (Gateway® INVITROGEN) together with the donor plasmid pDONR221. After sequencing *R3aGH* ORF variants were introduced in pK7WG2 (Karimi et al. 2002) by LR reaction (Gateway® INVITROGEN).

Agroinfiltration and PVX agroinfection assays

Recombinant *A. tumefaciens* strains were grown as described previously (Champouret et al. 2009), except that culturing steps were performed in LB media supplemented with 100 µg/ml kanamycin for the pGR106 and pGRAB constructs, and 100 µg/ml spectinomycin for the pK7WG2 constructs. Agroinfiltrations were performed on the middle section of leaf panels of *Solanum* plants beyond nine-leaf stage, or four to five week old *Nicotiana benthamiana*. Plants were infiltrated with the *A. tumefaciens* suspensions, and symptoms were monitored at two to six day after infiltration (dai). Plants were grown and maintained throughout the

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experiments in a greenhouse with an ambient temperature of 22–25 °C and high light intensity. For transient co-expressions *A. tumefaciens* solution were mixed in a 1:1 ratio before infiltration. PVX agroinfection was performed as described in previous studies (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers et al. 2006; Vleeshouwers and Rietman 2009).

Table S1: List of used primers.

Primer name	Primer sequence 5'- 3'	Comments
Pex147F	CCATGCGTCTGGCAATTATGCT	Avr3a variants amplification
Pex147R	CTAATATCCAGTGAGCCCCAGGTG	
R3a-GatBP-F2	GGGGACAAGTTTGTACAAAAAAGCAGGCTATG GAGATTGGCTTAGCAGTTGGTG	R3a gene homologues amplification
R3a-GatBP-R2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCA CATGCATTCCCTATCGATCTT	
SH194-2-GatBP-R2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCA GATTGGATATGGCAAGTGTTC	
R3a-Gat-F	CACCATGGAGATTGGCTTAGCAGTTGGTG	R3a gene homologues sequencing
R3a-Gat-R	TCACATGCATTCCCTATCGATCTT	
SH194-2-GatBP-R2-G	GCTGGGTTCAGATTGGATATGGCAAGTGTTC	
R3aH-307-29F	CAGCATCAAAATCTTGCAGAAAC	
R3aH-607-28F	GTAGTTCCTATTGTTGGAATGG	
R3aH-961-82F	GAGAGTGTTGCCTTGATGATGG	
R3aH-1202-23F	GAAGTGAAATATGGGAGCTGCC	
R3aH-1501-22F	CATGACCTTGTCAATGATTTAG	
R3aH-1934-54F	TCTTGAGGAGCTACCGCTGCA	
R3aH-2244-65F	CAATTCACAAACAGAAAGAGAC	
R3aH-2592-12F	GGAGAGTTCCCTATACTTGAG	
R3aH-2913-37F	CAACCTTACTAGGTTTTTGATTCC	
R3aH-3446-70F	ACCTCACTTCGCTTCAAAGTCTAC	

Sequences underlined correspond to Gateway® (INVITROGEN) attB1 and attB2 sites.

Phylogenetic data analyses

Phylogenetic analyses of the R3a gene homologues in *S. stoloniferum* 554-2 (StoR3aGH) were conducted using the Neighbour-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). R3a, I2GA-SH23-1, I2GA-SH23-3, I2GA-SH194-2 from SH83-92-488 *S. tuberosum* and I2 from tomato were used as closest known sequences and the tree was rooted using Rpi-BLB1. Bootstrap values greater than 50% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by mega. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences.

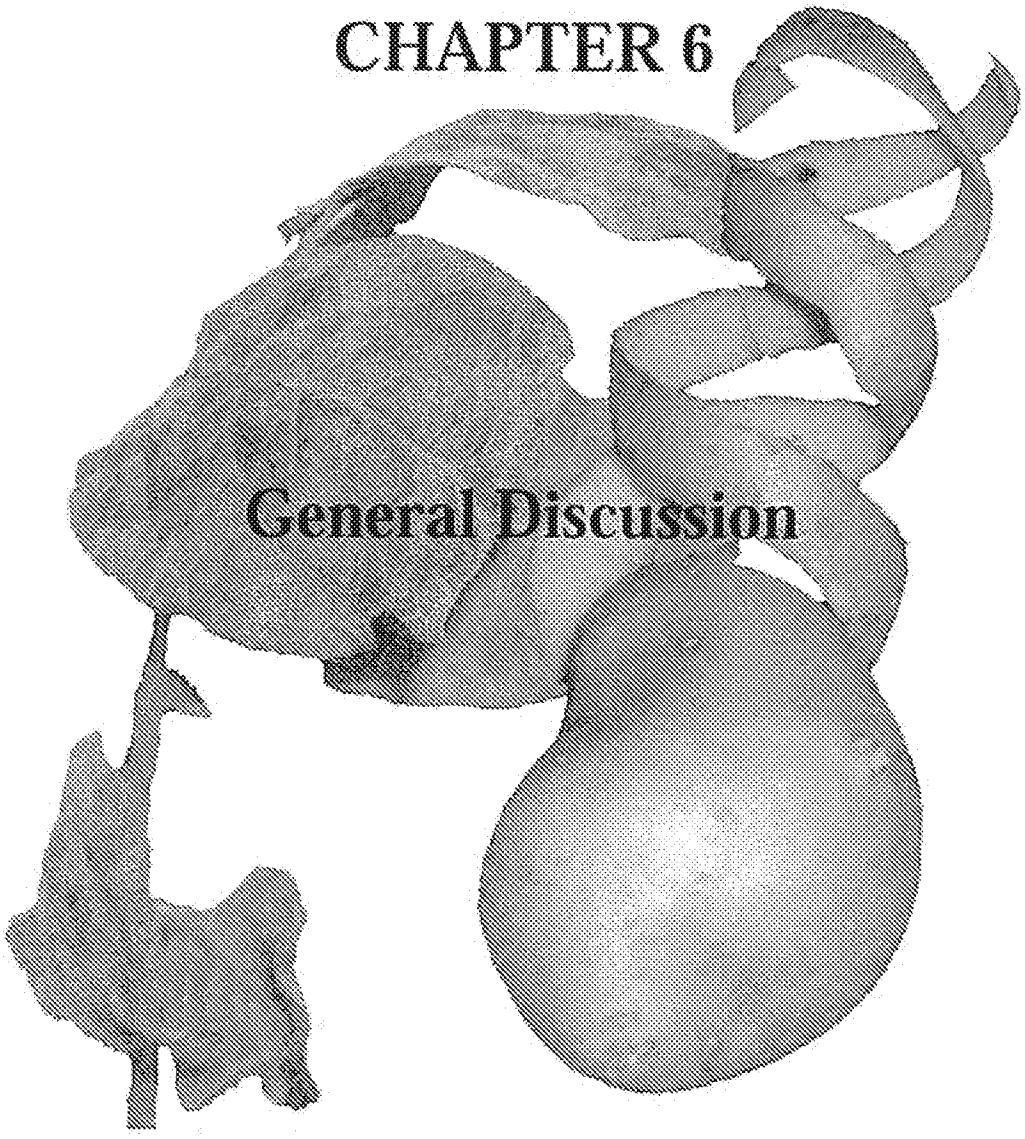
Acknowledgments

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CHAPTER 6



General Discussion

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Introduction

This thesis, entitled "Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes" was aimed to identify new R/AVR pairs and functionally characterize the *R* gene as well as the cognate *Avr* gene. Knowledge of *R* and *Avr* as they evolved under natural conditions can then be applied to explain or predict the performance of *R* genes in natural and practical situations. To answer such questions we have based our approaches on effector genomics, allele-mining of *Avr* and cognate *R* genes pairs, accurate disease testing with well-characterized *Solanum* genotypes, transient complementation assays, and a diverse set of *P. infestans* isolates from different geographic origin and with distinct lineage based on SSR markers. This discussion will reflect on the experimental findings described in this thesis, their impact for future management of late blight and new questions.

Effectoromics potential

Oomycete plant pathogens, such as *P. infestans*, secrete an arsenal of effector proteins that modulate host innate immunity and enable parasitic infection (Birch et al. 2009; Birch et al. 2008; Haas et al. 2009; Kamoun 2006; Schornack et al. 2009b). Host immunity responses are initiated by a number of these effectors, namely members of the RXLR family, by triggering hypersensitive cell death and resistance responses through activating particular NBS-LRR immune receptors or resistance (*R*) genes, generically known as CC-NBS-LRR in the cytoplasm (Dangl and Jones 2001).The transient assay based on *Agrobacterium tumefaciens* carrying a binary vector made of Potato Virus X of which the virulent gene is replaced by *P. infestans* C-terminal domain of an effector (PVX agroinfection) proved to be a useful high throughput system to screen for resistance responses in wild *Solanum* species (Vleeshouwers and Rietman 2009). In Chapter 5, the agroinfection approach in combination with agroinfiltration and disease assays provided an efficient platform for *in planta* testing of large numbers of genotypes with *R3a* gene homologue (*R3aGH*) candidates and led to the identification of one functional *R3aGH* in *S. stoloniferum*. In addition, we identified nine other genotypes from Mexico and Argentina that contain a putative functional *R3aGH*. In Chapter 3, we have identified three wild *Solanum* species (*S. edinense*, *S. schenckii* and *S. hjertingii*) responding to the RXLR effector PEXRD11, which is related to a previously described RXLR effector gene *PiAvr2* (Lokossou et al. 2009). Genetic studies demonstrated that the new *Rpi*-candidates responding to PEXRD11 in *S. edinense* and *S. schenckii* was located in the *R2* locus. Using the effector again we could rapidly test a number of *R2* gene homologues (*R2GH*) for cell death activity with PiAVR2/PEXRD11 in agroinfiltration assays. We efficiently identified six new R2GH that induced cell death with PEXRD11 and PiAVR2, and all six R2GH confer resistance to *P. infestans* isolate avirulent on R2-plants.

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The *R* genes were designated *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*. In Chapter 4, we continued on the same R-AVR interaction and mined 11 related effectors of the *PiAvr2/PexRD11* effector family from the *P. infestans* T30-4 genome (Haas et al. 2009). This observation of high diversity of genes in this effector family is explained by the highly accelerated rate of evolution of effectors (Hogenhout et al. 2009). Challenging independently the 13 effectors of the PiAVR2/PEXRD11 family with the R2GH cloned in chapter 3 and the already known functional resistance genes *R2*, *R2-like*, *Rpi-abpt* and *Rpi-blb3* (Lokossou et al. 2009), resulted in the identification of two effectors, PITG_21949 and PITG_21645, with avirulence activity against the ten functional R2GH. Furthermore, we identified a differential recognition between the six new R2GH of chapter 3 and the four known R2GH for PITG_13940. In all these studies, effectors are the key to characterise the R2GH response spectrum.

One difficulty we encountered with the PVX agroinfection technique is the extreme resistance (ER) phenotype (Chapter 5). Extreme resistance is seen with a number of *R* genes conferring resistance to viruses; it is manifested as a complete lack of both macroscopic and microscopic HR lesions and is accompanied by the absence of detectable virus accumulation (Bendahmane et al. 1999; Kang et al. 2005; Kohm et al. 1993). However interpretation of results can be difficult since many *R* genes that induce ER, including *Rx*, also induce HR depending on the strain of virus, host genetic background and environmental conditions (Baurès et al. 2008; Bendahmane et al. 1999; Kang et al. 2005). We observed these HR and ER responses and this posed a few questions. The response pathway in R/AVR interaction between R3a and $AVR3a^{KI}$ has always been observed by HR responses with agroinfiltration assay, but in this study we have observed ER on R3a plants when infected with PVX-$AVR3a^{KI}$. Recently, Liu et al. (2010) demonstrated that HR is dispensable for R protein-mediated induction of systemic acquired resistance (SAR), and that Rx-induced SAR is mediated by the same salicylate-dependent pathway induced by other R proteins, even if it results in ER response. Therefore, future studies to understand the switch of phenotype observed for R3a/$AVR3a^{KI}$ interaction could bring new solutions for resistance breeding by providing *R* genes with a fast resistance response induction which would stop infection not only from avirulent isolates but also from avirulent and aggressive isolates, which sometime escape from the HR response (Chapter 2).

In conclusion, effectoromics has been a successful approach to identify R-AVR interactions with new RGHs and AVRs. Moreover we have proven that in combination with accurate disease tests based on well-characterized *P. infestans* strains in *Solanum* and *N. benthamiana* we could functionally characterise the RGHs. These results, therefore, offer a great future for effectoromic studies in fundamental and applied sciences (Schornack et al. 2009b).

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Functional characterisation of *R* genes

Since the discovery that single *R* genes in potato were quickly defeated by virulent *P. infestans* isolates in the field, potato breeders focussed their search for resistance on *R* genes that are effective against a broad range of *P. infestans* isolates, preferably against all isolates. Two *R* genes against late blight, *Rpi-blb1* and *Rpi-blb2*, from the Mexican *S. bulbocastanum*, were originally described as broad spectrum *R* genes, since there were no reports of defeat by highly virulent *P. infestans* isolates (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Interestingly, the qualification of broad spectrum was defined by only three and two "avirulent" isolates from The Netherlands and Belgium in the case of *Rpi-blb1*, and *Rpi-blb2*, respectively. In Chapter 2 we reassessed the resistance spectrum of *Rpi-blb1* using a broad diversity of *P. infestans* strains from Europe and Mexico, including isolates that were isolated from *S. stoloniferum* of which the *Rpi-blb1* homologue *Rpi-sto1* was recently isolated (Vleeshouwers et al. 2008; Wang et al. 2008). Two isolates, PIC99189 and PIC99177 collected in 1999 from the Toluca Valley in Mexico were infecting *Rpi-blb1*-plants, and the myth of *Rpi-blb1* being a broad-spectrum *R* gene to all *P. infestans* isolates, was defeated. Studying the genetic diversity of *Avr-blb1* (*ipiO*) (Pieterse et al. 1994; Vleeshouwers et al. 2008) revealed that *ipiO* is highly diverse, and that class 1 ipiO was the most abundant class in most European isolates. This explains why *Rpi-blb1* had been performing well against most isolates in Europe. Interestingly, we found that virulent isolates on *Rpi-blb1*-plants are lacking *Avr-blb1* Class 1 ipiO, and these evolved in Mexico (Chapter 2). These findings suggest that migration of isolates from Mexico, where *S. stoloniferum* occurs, to European farming areas, will probably lead to defeat of *Rpi-blb1* in Europe. Similar phenomena already happened with A2 mating type of *P. infestans* isolates was found in Europe in the 1980s (Goodwin and Drenth 1997; Hohl and Iselin 1984; Spielman et al. 1991). Also, directional selection for virulence is superimposed when race-specific resistance is introduced (Montarry et al. 2006), and therefore, spread of virulence against *Rpi-blb1* is just a matter of time. In addition, deployment of *Rpi-blb1* as a single *R* gene in potato is expected to even accelerate the process.

In another effector genomics study, the cognate *Avr* gene matching *Rpi-blb2* was identified. Phylogenetic analysis by maximum likelihood showed that *Avr-blb2* (*PexRD39/40*) is highly diverse in the *P. infestans* population. One variant, named PEXRD40^{V69F}, with a.a. in position 69 being a phenylalanine, was identified to be not recognized by *Rpi-blb2* (Oh et al. 2009). Since the *P. infestans* genome showed presence of 7 copies of *Avr-blb2* this may decrease the possibility to have this virulent variant in all seven positions, and that could explain to some extent the degree of the durability of this resistance gene in the field. Still, virulent isolates on *Rpi-blb2* plants were discovered recently in potato trap fields (Dr. G. Kessel, Biosciences, Wageningen University and Research centre, personal communication), and these isolates are currently being studied to understand how virulence

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on *Rpi-blb2* plants can occur. One hypothesis is perhaps a combination of presence of virulent variants of Avr-blb2 and silenced avirulent variants as observed for *Avr-vnt1* in a *P. infestans* isolate that is virulent on *Rpi-vnt1* plants (Pel 2010). The flexibility of the *P. infestans* genome can result in various ways to escape recognition by resistant plants, and characterisation of each individual R/AVR is important prior release of new resistance genes in the field.

Another important aspect to take into consideration regarding the description of an *R* gene against late blight is the efficiency of the *R* gene when exposed to aggressive isolates. Frequently, aggressiveness is interpreted as virulence (Suassuna et al. 2004). Niks (2001) highlighted the discrepancies between terminologies of virulence in a review. De Wit (2000) described virulence based on a functional gene that determines quantitatively or qualitatively the disease development, and therefore the term virulence described as the loss of avirulence should be avoided. However, Boller and Keen refer in the same multi-author book virulence as to the loss of avirulence, based on the gene-for-gene model (Boller and Keen 2000). The first description of virulence, as a determinant of quantitative or qualitative disease development, does not make any difference between effector genes that have a virulence function to the pathogen by suppressing the host defence system, for example, AvrPto and AvrPtoB which target the kinase domains of the receptor-like kinases CERK1, BAK1, EFR1 and FLS2 in *P. syringae* (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008), and metabolic genes involved in growth development or life cycles of the pathogen, such as a transcription factor with a highly conserved APSES domain of *Ustilago maydis* which regulates dimorphism, sporulation, and pathogenic development (2010). Here, we follow the definition of aggressiveness as the overall ability of a pathogen to infect its host plant (Chacon et al. 2007; Cooke and Deahl 1998), irrespective of specific virulence or avirulence determinants and more based on physiological and metabolic pathway fitness. In Chapter 2 we have demonstrated that an aggressive isolate of *P. infestans*, PIC99183, could overrule the recognition by *Rpi-blb1*-plants, with 50% infection efficiency but with a low lesion growth rate, despite the fact that this isolate contains a class I *ipiO* variant. Aggressive isolates were also able to infect transgenic *Rpi-blb1*-plants which had one or two copies of the resistance gene, even if these isolates were expressing avirulence alleles. In contrast, aggressive isolates could not infect wild *S. bulbocastanum* that express *Rpi-blb1* in their own genetic context at higher levels, compared to transgenic potatoes. Our data are all in line with recent studies that suggested a positive correlation between resistance level of *Rpi-blb1* plants and *Rpi-blb1* expression level (Bradeen et al. 2009; Kramer et al. 2009). Therefore, insufficient expression levels of *R* genes in the field can also lead to susceptible phenotypes when challenged with aggressive isolates of *P. infestans*.

In conclusion, to describe and predict the range of resistance of *R* genes against late blight, characterisation of genetic diversity and expression of the corresponding Avr gene in *P. infestans* isolates is essential. In addition, the level of *R* gene transcript that is required to

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confer resistance when challenged by aggressive and contemporary isolates of *P. infestans* has to be characterized.

Co-evolution of R loci and their corresponding AVR loci

R genes are classified in two rates of evolution. Type I *R* genes are characterized by frequent sequence exchanges between paralogs that obscure orthologous relationships, whereas type II resistance genes rarely undergo sequence exchanges with paralogs and maintain orthologous relationships (Friedman and Baker 2007; Kuang et al. 2004). This thesis presents three different R-AVR pairs involved in the co-evolution between the *Solanum* section *Petota* host species and *P. infestans*, namely R3a - AVR3a (Chapter 5), Rpi-blb1 - AVR-blb1 (Chapter 2) and R2 -PiAVR2/PEXRD11 (Chapter 3 and 4).

R3a is located in loci known for hot spot recombination (Huang 2005; Huang et al. 2005) and fits perfectly to the Type I *R* gene definition. In Chapter 5, we detected 29 R3aGH among which one was functional with $AVR3a^{KI}$ in *S. stoloniferum*. However, all *stoR3aGH* still have a high homology to *R3a* and its paralogues, and therefore are likely to evolve by sequences exchanges in this hot-spot, in line with the Trench-Warfare-model (Stahl et al. 1999). On the pathogen side there are only three variants of the effector AVR3a detected thus far (Armstrong et al. 2005). In the co-evolution dynamics, this high diversity on the resistance gene side combined with the low diversity on the effector side could fit to an effector-triggered immunity (ETI) step in the Zig-Zag model of Dangle and Jones (2006), where one effector is recognised and host develops a high diversity of RGHs in an attempt to recognise all variants of the effector.

In Chapter 3 we discovered that the R2 cluster is highly diverse and with other studies (Lokossou et al. 2009), we defined the R2 cluster also as a Type I R gene. Interestingly, effectors recognized by functional R2 homologues are allelic variants that are present as multiple copies in the genome and belong to two different gene structures, PiAVR2 and PEXRD11, based on a.a. alignment (Chapter 4). In the case of *S. bjertingii* we demonstrated the presence of four functional R2GH with different spectrum of recognition to five PiAVR2/PEXRD11 members (Chapter 3 and 4). Bergelson et al. (2001)) and Dawkins and Krebs (1979) refer to a classical arms race as a series of selective sweeps as novel *R*-gene alleles, capable of recognizing pathogenicity determinants (*Avr*) that previously avoided detection in a plant population, spread to high frequency. The longevity and high allelic diversity in some cases suggest a micro-evolutionary mechanism that promotes the maintenance of stable polymorphism (Stahl et al. 1999) until the pathogen overcomes the resistance (Jones and Dangl 2006). Therefore, we argue that *R2-PiAvr2/PexRD11* with multiple copies of each gene family in the genome of the host and in the genome of the pathogen fits to the arms-race model (Bergelson et al. 2001; Dawkins and Krebs 1979). This

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diversity on the *R* loci and the *Avr* loci could represent an advanced stage of co-evolution between the host and the pathogen, where multiple cycles of resistance (ETI) followed by susceptibility (ETS) have occurred in the Zig-Zag model.

In Chapter 2 we worked with a type II *R* gene (*Rpi-blb1*) which is present in resistant genotypes and just absent from its locus in susceptible genotypes (van der Vossen et al. 2003). Moreover little genetic diversity has been found in wild *Solanum* species for *Rpi-blb1* and only three functional variants of Rpi-blb1 have been identified so far (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008; Wang et al. 2008). The low diversity of Rpi-blb1 can be explained by having another role than just late blight resistance (van der Vossen et al. 2003), or by representing a recent *R* gene which has not (long) been under co-evolutionary pressure yet. The latter could explain why no susceptible variants of *Rpi-blb1* were found in this locus (van der Vossen et al. 2003) or only one form with 18bp deletion (Song et al. 2003) scattered around in *S. bulbocastanum* species only in Central America (Lokossou et al. submitted; Vleeshouwers et al. 2008; Wang et al. 2008). In contrast, on the effector side we identified up to 13 variants of *Avr-blb1* in *P. infestans*. This might reflect a selection pressure to escape recognition by *Rpi-blb1*. Therefore, we postulate that the natural co-evolution described by this interaction might be in the first transition between effector-triggered immunity (ETI) and effector-triggered susceptibility (ETS) (Jones and Dangl 2006).

In conclusion, the different R/AVR pairs presented in this thesis seem to reflect a variety of co-evolutionary stages and together provide more insight in the genetic coexistence mechanism of potato and *P. infestans*.

The future of resistance breeding to late blight

Potato breeding for late blight resistance was one of the earliest practices mankind used to combat plant pathogens by means of genetic improvement. Introgression of resistance genes from wild *Solanum* species into potato cultivars in the past has only provided temporary solutions and durable resistance based on one single R gene appears nowadays more and more an illusive theory. The genome flexibility of *P. infestans* as well as its fast evolving abilities (Fry 2008; Haas et al. 2009) are two of the reasons that explain the fast defeat of *R* genes in agricultural practice (Hein et al. 2009). The recent molecular insight in biology of the pathogen (Birch et al. 2009; Birch et al. 2008; Birch et al. 2006; Kamoun 2007; Morgan and Kamoun 2007; Tyler 2009; Whisson et al. 2007; Win et al. 2007), the better understanding of the interaction with the host based on co-evolutionary principles (Armstrong et al. 2005; Lokossou et al. 2009; Oh et al. 2009; Pel 2010; Vleeshouwers et al. 2008; Chapter 3 and 4), the newly uncovered annotated *P. infestans* genome (Haas et al. 2009), and the development of new techniques to functionally characterise the gene pool of

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effectors of the pathogen (Oh et al. 2009; Vleeshouwers et al. 2008), provide new genetic tools to manage the disease. In this thesis on effectoromics we have characterized new RGHs, some of which with new functionality, and we have uncovered the diversity of *Avr-blb1* in *P. infestans* population. Below we present the utilities of these results in future potato breeding programs, and discuss some new research areas of interest for future *R* gene characterisation.

We demonstrated in Chapter 3 that effectoromics accelerates the cloning of functional *R* genes. The screening of *Solanum* germplasm with effectors can provide a pattern of responses to a specific effector from a group of *Solanum* species, and therefore, can help to avoid redundant *R* gene cloning efforts. For these reasons, future studies to identify the recognition spectrum of one *R* gene in the wild *Solanum* population should not only be based on the resistance spectrum to *P. infestans* isolates but preferably on the responses to the cognate effector.

Effectoromics also accelerates the characterization of functional *R* genes, and identification of additional RGH can lead to a broader resistance. For example, six functional R2GH respond to PITG_13940 of *P. infestans*, whereas the four already known R2GH do not. Interestingly in *S. bjertingii* we have found that three R2GH are expressed, i.e. *R2-like* responding to PiAVR2, PEXRD11, PITG_21949 and PITG_21645, and *Rpi-bjt1.2* and *Rpi-bjt1.3* with additional response to PITG_13940. This supplementary specificity of recognition can be valuable for potato breeding and by combining R2GH get a broader spectrum of resistance against *P. infestans*. However before using these new R2GH in breeding programs it would be of interest to study the expression level of these five effectors from the *PiAvr2/PexRD11* family in contemporary *P. infestans* populations.

Knowledge on R/AVR pair diversity, like in the Rpi-blb1/AVR-blb1 interaction provides new handles for late blight management. Durability of an *R* gene is based on its application. Therefore the large-scale monitoring aimed at diagnosing the *Avr-blb1* classes in the *P. infestans* population on commercial potato growing areas can be applied to detect (evolution and migration of) virulence against *Rpi-blb1*. When virulence is detected selection pressure towards losing class 1 *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and applying other *R* genes instead (Chapter 2). In conclusion, one way to manage the selection pressure on the pathogen is by large scale effector monitoring for virulence frequencies in the *P. infestans* population

Mapping and cloning of new *Rpi* genes is mainly done on wild *Solanum* genotypes collected in Central and South America 50 or more years ago (*Sol*Rgene database). However, the high genetic variability in the tetraploid potato genome (D'Hoop et al. 2010; Simko et al. 2006), which is even more important in *R* gene loci, shows that genetic changes occur at high rates. For example, a SNP every 15 bp in the region of the *StVe1* resistance gene against *Verticillium albo-atrum* was identified by Simko et al. (2004). Also, *R* loci contain multiple transposon families which are though to be involved in the rapid diversification (Song et al. 1997). Recent evidence that infection may stimulate host recombination rates in subsequent

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generations via induction of transposable elements (Mirouze et al. 2009; Molinier et al. 2006), and demonstration of instability of an epigenome (Reinders et al. 2009), allows us to extrapolate that contemporary wild *Solanum* species may display new resistance gene that are more adapted to the contemporary *P. infestans* population. As a result, the continuation of collecting wild *Solanum* species in centers of co-evolution such as the Toluca Valley and South America is of great interest for future breeding programs.

To conclude, intensive farming aims to create a crop without any diseases. In the natural environment this is an utopia, all plants coexist with their pathogens and a co-evolutionary balance has been created which allows both partners to be still alive even up to this date. New insight in this co-evolution mechanism show that presence of both partners is needed to create new natural *R* genes and matching *Avr* effectors. As a result and due to the recent constrain to reduce use of pesticides, modern potato farming should aim more for (i) integrating pest management by monitoring virulence of *P. infestans* contemporary population, (ii) continue screening contemporary wild *Solanum* species for new resistance genes that have co-evolved with the pathogen, also in the last decades, and (iii) integrate the natural high degree of diversity of functional *Rpi* genes in stacking approaches or in *in vitro* evolution approaches like shuffling techniques (Farnham and Baulcombe 2006), to create new *R* genes with altered specificities.

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Summary

Potato (*Solanum tuberosum L.*) is nowadays the most important non-cereal food crop in the world. It is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification, isolation and characterization of *R* genes. Since the genomics era, the *P. infestans* genome is disclosing numerous effector genes. Effectors are molecules that manipulate the host to facilitate infection and/or trigger defence responses. In the presence of a matching *R* gene, effectors cause a hypersensitive response (HR) that often leads to avirulence, and in such case they are designated avirulence (*Avr*) genes. One group of cytoplasmic effectors of *P. infestans* is characterised by an RXLR motif which allow these proteins to be translocated in the host cytoplasm. This thesis employs effectoromics, use of effectors to probe resistance in a host plant, by using RXLR effectors of *P. infestans* to probe resistant *Solanum* germplasm for late blight *R* genes and sort out their functional redundancy and specificity. Research was focused on the genomic diversity and (co-)evolution of three R/AVR interactions: (i) the *Rpi-blb1/ipiO* pair by exploring the genetic diversity of the *ipiO* effector family in a large set of *P. infestans* isolates, (ii) the *R3a/Avr3a* pair, with the identification of various *R3a* gene homologues (*R3aGH*) and a functional *R* gene (*Rpi-sto2*), and (iii) the *R2-PlAvr2/PexRD11* interaction, where we identified and characterized genetic diversity at both *R* and AVR side, and describe their co-evolution.

In the last half century, most late blight *R* genes that were introgressed in potato were quickly defeated. In the early XXI century, a Resistance gene against *Phytophthora infestans* (*Rpi*-gene) from *S. bulbocastanum* (*Rpi-blb1*) was characterized as an exclusive broad-spectrum *R* gene. Using the recently uncovered avirulence effector *Avr-blb1* (also known as *ipiO*) recognised by *Rpi-blb1*, we have screened and characterized a genetically diverse set of *P. infestans* isolates originating from different countries, for their *ipiO* diversity (**Chapter 2**). In total 16 *ipiO* variants grouped in three distinct classes by phylogeny were identified. Class I and II, but not class III *ipiO* variants induced cell death when co-infiltrated with *Rpi-blb1* in the model plant *Nicotiana benthamiana*. The major breakthrough came from the discovery of the two first *P. infestans* isolates that defeated *Rpi-blb1*. The virulence on *Rpi-blb1* potatoes correlated with the absence of the highly diverse class I *ipiO*. Both isolates were originating from Mexico, one of the centres of origin of the pathogen, and probably evolved due to the resistance pressure of the host in this region. Consequently, the expected broad-spectrum *Rpi-blb1* has shown weaknesses as any other *R* gene against late blight. This study showed that characterization of the genetic diversity of an *Avr* gene is essential to predict the effectiveness of an *R* gene in long-term disease management. Moreover, our findings provided a technical solution for potato farmers, large-scale monitoring aimed at diagnosing the *Avr-blb1* classes

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in the *P. infestans* population on commercial potato growing areas can be applied to detect (evolution and migration of) virulence against *Rpi-blb1*. When virulence is detected, cultivars with *Rpi-blb1* should be omitted for a certain period of time and other *R* genes applied in stead, in order not to increase selection pressure towards losing class I *IpiO* in the pathogen.

In an attempt to identify new *Rpi* genes against late blight we have screened and probed resistant *Solanum* germplasm with cytoplasmic effectors of *P. infestans* using a transient assay based on Potato Virus X (PVX) agroinfection (**Chapter3**). This approach resulted in the identification of candidate *Avr* gene *PexRD11*. Further screening with PEXRD11 on a large set of genotypes representing the broad *Petota* genepool, followed by mapping studies, resulted in the identification of new *Rpi*-candidate genes against *PexRD11* in the *R2* cluster. Interestingly, only three species originating from Mexico responded to PEXRD11, i.e. *S. edinense*, *S. schenckii* and *S. hjertingii*. Allele mining on these genotypes yielded 17 new *R2* variants grouped in six classes. From these, six new *R2* homologues were characterized, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3*, recognising *PexRD11* and *PiAvr2*, a previously described related RXLR effector gene, and conferring resistance to *P. infestans*. PEXRD11 and PiAVR2 have a low percentage of similarity, however some degree of redundancy was observed because both are triggering cell death when co-infiltrated with R2 homologues. We concluded that the *R2* family has evolved in Mexico, one putative centre of origin of the pathogen, and provided an overview of the evolution and functional diversification of this *R* gene family in its natural environment.

The recent completion of the sequence and annotation of the *P. infestans* genome provides new opportunities to study the evolution of gene families of this pathogen and new avirulence functions for some of its effectors can be identified. Following the discovery of the effector PEXRD11 recognition by R2 in Chapter 3, research was focussed on the identification and characterization of *PiAvr2/PexRD11* family members within the *P. infestans* genome (**Chapter 4**). In addition of *PiAvr2* and *PexRD11*, 11 new independent RXLR effectors were identified that were grouped in three classes. A robust association was discovered between phylogenetic classes of the PiAvr2/PexRD11 effector family and the R2 resistance gene family with recognition leading to cell death. The ten known functional R2 homologues, i.e. the six found in Chapter 3 and the already cloned *R2*, *R2-like*, *Rpi-abpt* and *Rpi-blb3*, recognised three new effectors, i.e. PITG_13940, PITG_21049 and PITG_21645, with different specificity. The six R2 homologues responded to PITG_13940 but not the four previous known R2 homlogues which were used in breeding material for the last decades. Different hypotheses based on secondary structure, and presences of motifs between the PiAVR2/PEXRD11 effector family members have been discussed to explain induction or absence of cell death with the ten R2 homologues. All together, these results highlight the diversity of the R2-PiAVR2/PEXRD11 interaction resulting from a co-evolution of the *Solanum*/*P. infestans* pathosystem. Moreover by using effectoromics, six new *R2* homologues variants with potentially a broader resistance spectrum could be characterized.

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This study therefore provides more diversity and specificity of *R* gene homologues, which may be valuable for potato breeding.

R3a from *Solanum demissum* is known to interact with the avirulent effector $Avr3a^{KI}$ of *P. infestans*, resulting in the induction of cell death visible by necrotic symptoms on the interaction site. Screening wild *Solanum* species to identify R3aGH and new R genes responding to the virulent allele $Avr3a^{EM}$ and/or avirulent allele $Avr3a^{KI}$ is crucial to provide more *R* gene diversity, as we saw in Chapter 4. In **Chapter 5** we have combined three different techniques to gradually reduce the number of genotypes which potentially contained a functional R3aGH. On the 177 genotypes tested, we indentified nine genotypes with the same pattern of response to the effectors as three R3a-plants and with seven of them showing the same resistance spectrum as R3a plants. Interestingly these genotypes came from two different centres of diversity, Mexico and Argentina. Allele mining on one of the seven genotypes, *S. stoloniferum* from Mexico, revealed a high rate of evolution for *R3a*. We identified one functional *R3aGH*, designated *Rpi-sto2*, which triggered cell death when coinfiltrated with $AVR3a^{KI}$ on *N. benthamiana*. This study shows that *R* genes in the *R3a* locus are under a tremendous evolutionary pressure from the pathogen with the presence of only one functional *R3aGH* out of 29 variants cloned in the Mexican *S. stoloniferum*, and that *R3a* has potentially evolved under pressure of two geographically distinct populations of *P. infestans*. To unravel the tight pressure from the pathogen on this resistance gene we monitored the *Avr3a* family allelic combination in a set of isolates of *P. infestans*. We observed an over-representation of isolates that only contained virulence allele $Avr3a^{EM}$, which suggests a selection pressure to avoid $AVR3a^{KI}$ recognition in host plants. In addition we discovered a new allele, $Avr3a^{KIL}$, which thus far seems to have similar specificity as $Avr3a^{KI}$. Our results in this chapter offer new *R3aGHs* that can be used in future breeding programs or in molecular engineering of *R* genes with perhaps a broader spectrum of effector recognition.

This study has led to the development of genetic tools to identify and characterise new RGHs valuable for potato breeding. Moreover the functional genomics studies of specific R/AVR pairs of *Phytophthora infestans* effectors and *Solanum* resistance genes have brought more insight in the co-evolution of this pathosysthem. In conclusion, three major discoveries have been made, 1. Discovery of new *R* gene homologues for *R2* and *R3a*, 2. With the *R2* homologues displaying a broader spectrum of responses to the *PIAvr2/PexRD11* family members of *P. infestans*, and 3. Characterization of the diversity of *ipiO* variants in the *P. infestans* population, which can be used for monitoring and provides new perspectives for management of *Rpi-blb1* expressing cultivars in the field.

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Samenvatting

De aardappel (*Solanum tuberosum* L.) is wereldwijd het belangrijkste voedselgewas, na tarwe en rijst. Echter, de aardappelteelt lijdt jaarlijks grote verliezen door de aardappelziekte, die veroorzaakt wordt door de oömyceet *Phytophthora infestans*. Voor het beheersen van de aardappelziekte tijdens de teelt zijn gestapelde resistentiegenen (*R* genen) nodig. Om deze te verkrijgen zijn nieuwe strategieën voor de identificatie, isolatie en karakterisering van R genen een vereiste, en hier worden nieuwe mogelijkheden beschreven die dit op een efficiënte manier mogelijk maken. Tijdens het huidige "genomics" tijdperk, zijn in het genoom van *P. infestans* vele effectorgenen ontdekt. Effectors zijn moleculen van *Phytophthora* die de waardplant manipuleren om infectie mogelijk te maken, en/of afweerreacties te initiëren. Als een effector en het bijbehorende *R* gen beide aanwezig zijn, wordt in de plant de overgevoeligheidsreactie (HR) geïnitieerd. Dit leidt vaak tot avirulentie (de plant is resistent), en in dit geval noemen we de effectors ook wel avirulentiegenen (Avr). Een groep van cytoplasmatische effectors van *P. infestans* bezit een zogenaamd RXLR motief, dat ervoor zorgt dat de effectors de plantencel binnen kunnen dringen om daar hun (a)virulentie rol te vervullen. Dit proefschrift beschrijft "effector genomics", het gebruik van effectors om resistentiegenen te identificeren in resistente planten. We testen RXLR effectors op wilde *Solanum* soorten voor het induceren van HR, om daarmee de aanwezigheid van *R* genen te ontdekken. Vervolgens karakteriseren we de *R* genen op hun werking met behulp van functionele assays. Ons onderzoek was gericht op genetische diversiteit en de (co-)evolutie van drie specifieke *R / Avr* interacties: (i) de *Rpi-blb1 / ipiO* interactie, waarbij de genetische variatie van de *ipiO* familie bestudeerd is in een uitgebreide set van *P. infestans* isolaten, (ii) de *R3a / Avr3a* interactie, waarbij een aantal *R3a* homologen (*R3aCH*) en een nieuw, functioneel, *R* gen (*Rpi-sto2*) geïdentificeerd zijn, en (iii) de *R2 / PiAvr2/PexRD11* interactie, waarbij de genetische diversiteit aan zowel *R* als *Avr* gene gekarakteriseerd is. Dit leidde tot een beschrijving van hun co-evolutie in Mexico.

In de tweede helft van de vorige eeuw, zijn ingekruiste *R* genen tegen *P. infestans* (*Rpi*) doorbroken waardoor resistente aardappelrassen toch weer vatbaar werden. In het begin van de 21e werd een nieuw *R* gen geïsoleerd uit *Solanum bulbocastanum*. Dit *Rpi-blb1* gen werd beschreven als een exclusief breed-spectrum *R* gen, dat duurzame resistentie zou geven. In **hoofdstuk 2** hebben we een diverse set van *P. infestans* isolaten gekarakteriseerd voor hun avirulentie eigenschappen op *Rpi-blb1*. We ontdekten twee *P. infestans* isolaten die *Rpi-blb1* konden doorbreken. Beide isolaten waren afkomstig uit Mexico, een gebied waar *P. infestans* oorspronkelijk geëvolueerd is, waarschijnlijk onder druk van resistente waardplanten uit die regio. Een studie naar de genetische variatie van *Avr-blb1* (ook bekend als *ipiO*) leverde 16 *ipiO* varianten. Deze konden, met behulp van een fylogenetische analyse, in drie klassen gegroepeerd worden. Klasse I en II *ipiO* bleken de HR te induceren na co-infiltratie met *Rpi-*

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blb1 in de modelplant *Nicotiana benthamiana*, maar klasse III *ipiO* deed dit niet. De virulentie van de nieuwe isolaten op *Rpi-blb1* aardappels hing nauw samen met afwezigheid van de zeer diverse klasse I *ipiO*. Deze resultaten bewijzen dat het *Rpi-blb1* gen, waarvan een breed-spectrum resistentie verwacht werd, inmiddels ook doorbroken is, net als eerder gebruikte *R* genen tegen *P. infestans*. Onze studie toont aan dat de karakterisering van genetische variatie van een *Avr* gen een goed handvat kan bieden om de werkzaamheid van een *R* gen in de aardappelplant te voorspellen. Dit is van belang voor resistentiemanagement op de lange termijn. Daarnaast bieden onze bevindingen een technische oplossing voor aardappeltelers: het op grote schaal monitoren van *Avr-blb1* klassen in de *P. infestans* populatie in commerciële aardappel velden is bruikbaar om ontwikkeling van virulentie tegen *Rpi-blb1* snel te detecteren. Zodra virulentie wordt gedetecteerd, zouden rassen met *Rpi-blb1* resistentie voor een zekere periode niet geteeld moeten worden, om de selectiedruk voor verlies van klasse I *ipiO* niet te stimuleren. In plaats van *Rpi-blb1* zouden dan tijdelijk aardappelrassen met andere *R* genen gebruikt kunnen worden.

In een poging om nieuwe *Rpi* genen en bijbehorende *Avr* genen tegen de aardappelziekte te identificeren, werden resistente *Solanum* soorten op het induceren van de HR tegen RXLR effectors van *P. Infestans* gescreend. Dit werd gedaan met behulp van een transiënte expressiemethode gebaseerd op het aardappel X virus (PVX) **(hoofdstuk 3)**. Deze benadering resulteerde in de identificatie van het kandidaat *Avr* gen *PexRD11*. Verdere screening met *PexRD11* op een grote set van aardappelgenotypen, die de brede "gene pool" van de *Solanum* sectie *Petota* (knoldragende wilde verwanten van de aardappel) representeren, resulteerde in de identificatie van nieuwe *R* gen kandidaten in het *R2* cluster. Slechts 3 *Solanum* soorten, allemaal afkomstig uit Mexico, reageerden op PEXRD11, t.w. *Solanum edinense*, *S. schenckii* en *S. hjertjingii*. "Allele mining" op deze genotypen leverde 17 nieuwe *R2* varianten op, die in 6 klassen werden gegroepeerd. Zes R2 homologen, t.w. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2 en Rpi-hjt1.3*, allemaal behorend tot klasse I of II, herkenden PEXRD11 en PiAVR2 (een eerder beschreven RXLR effector die herkend wordt door R2). PEXRD11 en PiAVR2 hebben weinig homologie, echter een zekere mate van functionele redundantie werd gevonden, omdat beide genen celdood induceren wanneer ze gecoïnfiltreerd worden met *R2* homologen. We concluderen dat de *R2* familie geëvolueerd is in Mexico, een van de waarschijnlijke oorsprongsgebieden van *P. infestans*, en presenteren een overzicht van de evolutie en functionele diversificatie van de R2 gen familie in zijn natuurlijke omgeving.

De recent beschikbaar gekomen, geannoteerde, *P. infestans* genoomsequentie biedt nieuwe mogelijkheden om de evolutie van genenfamilies te bestuderen, en nieuwe virulentiefuncties voor een aantal effectors te kunnen identificeren. Na de ontdekking van de PEXRD11 effector herkenning door R2 in hoofdstuk 3, hebben we ons gericht op de

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karakterisering van *PiAvr2 / PexRD11* genen families in het *P. infestans* genoom. Naast *PiAvr2* en *PexRD11* werden 11 nieuwe RXLR effectors geïdentificeerd en in 3 klassen gegroepeerd. Een robuuste associatie tussen het optreden van de HR en fylogenetische verwachtschap van de *Avr* en *R* familie werd gevonden. Tien bekende *R2* homologen, t.w. de 6 *R2* homologen beschreven in hoofdstuk 3 en de reeds eerder geklonseerde *R2*, *R2-like*, *Rpi-abpt* en *Rpi-blb3*, herkenden 3 nieuwe effectors, t.w. PITG_13940, PITG_21949 en PITG_21645, met verschillende specificiteit. De 6 R2 homologen gaven een HR na coinfiltratie met PITG_13940, maar de 4 reeds bekende R2 homologen die de afgelopen decennia in veredelingsprogramma's gebruikt zijn, deden dit niet. Verschillende hypothesen gebaseerd op de secundaire structuur van R2 genen, en aanwezigheid van bepaalde motieven in de PiAVR2/PEXRD11 varianten worden bediscussieerd om het al dan niet induceren van de HR met de 10 R2 homologen te verklaren. Samenvattend belichten onze resultaten de diversiteit van de R2 – AVR2/PEXRD11 interactie in het *Solanum* – *P. infestans* pathosysteem. Met effector "genomics" konden 6 nieuwe *R* gen homologen met een potentieel breder resistentie spectrum worden gekarakteriseerd. Deze studie laat additionele diversiteit en specificiteit van *R* gen homologen zien, die waardevol kan zijn voor resistentieveredeling van de aardappel.

Het R3a resistentie-eiwit uit *Solanum demissum* herkent het AVR3a^{KI} avirulentie-eiwit van *P. infestans*, en dit resulteert in de inductie van de HR. Om een bredere diversiteit van *R3a* homologen te identificeren in wilde *Solanum* soorten kunnen deze gescreend worden voor het optreden van de celdoodreactie tegen het avirulente allel Avr3a^{KI} en/of het virulente allel Avr3a^{EM}. In hoofdstuk 5 werden drie verschillende technieken gecombineerd, om gradueel het aantal genotypen met een potentieel functioneel R3a gen te reduceren. Van de 177 geteste *Solanum* genotypen, konden uiteindelijk 9 genotypen geïdentificeerd worden die dezelfde reactiepatronen gaven tegen de *Avr3a* effectors als 3 *R3a* controle genotypen. Zeven van deze genotypen lieten ook hetzelfde resistentiepatroon tegen *P. infestans* isolaten zien. De geïdentificeerde *Solanum* genotypen zijn oorspronkelijk afkomstig van twee verschillende oorsprongsgebieden, Mexico en Argentinië. "Allele-mining" op één van deze genotypen, *S. stoloniferum* uit Mexico, liet zien dat *R3a* snel evolueert. Tussen 29 *R3a* varianten vonden we een functionele *R3a* homoloog, *Rpi-sto2*, die de HR induceert na co-infiltratie met *Avr3a^{KI}* in *N. benthamiana*. Deze studie laat zien dat *R3a* onder hoge evolutionaire druk staat, en suggereert dat *R3a* mogelijk geëvolueerd is in twee geografisch verschillende gebieden met verschillende *P. infestans* populaties. Na bestudering van de allelische variatie van *Avr3a* in een aantal *P. infestans* isolaten vonden we dat isolaten met de *Avr3a^{EM}* variant oververtegenwoordigd waren, mogelijk door een selectie druk om AVR3a^{KI} herkenning in waardplanten te ontwijken. Naast de twee bekende allelen *Avr3a^{KI}* en Avr3a^{EM} vonden we nog een nieuw allel *Avr3a^{KI2}*, dat dusver dezelfde specificiteit blijkt te hebben als *Avr3a^{KI}*. Dit hoofdstuk biedt de ontdekking van nieuwe *R3a* homologen die direct gebruikt kunnen

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worden in toekomstige veredelingsprogramma's, of die via toekomstig gerichte moleculaire aanpassingen van *R* genen kunnen leiden tot een breder herkenningsspectrum van effectors.

Deze studie heeft geleid tot de ontwikkeling van nieuwe genetische handvatten voor het identificeren en karakteriseren van nieuwe *R* gen homologen die waardevol zijn voor de aardappelveredeling. Daarnaast hebben de effector "genomics" studies van specifieke R / AVR combinaties van *P. infestans* effectors and *Solanum* *R* genen meer inzicht gegeven in de co-evolutie van dit pathosysteem. Samengevat zijn de volgende waarnemingen gedaan, 1.) De ontdekking van nieuwe *R* gen homologen van *R2* en *R3a*, 2.) de ontdekking van *R2* homologen met een breder spectrum van resistentiereacties tegen *PiAvr2* / *PexRD11* familieleden, en 3.) de ontdekking dat genetische variatie van *ipiO* varianten gebruikt kan worden voor het monitoren van de *P. infestans* populatie en daarmee nieuwe perspectieven voor management van aardappelrassen met *Rpi-blb1* in de praktijk biedt.

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About the author

Nicolas Champouret was born on November 15th, 1978 in Dijon, Côte d'Or, France. After completing his academic high school in 1997, he started a BSc (1997-2000) in Cell Biology and Physiology at the University of Burgundy followed by a first year of MSc. (2000-2001) in Plant Biotechnology at the same University. He completed his MSc (2001-2002) at the University of Victor Segalen Bordeaux II on Fungal Biotechnology. During his second year of MSc, he performed a seven-month internship at the Department of Biology at the University of Fribourg, Switzerland, where he developed gateway vectors and techniques to transform the Oomycete pathogen *Phytophthora brassicae*. From 2002 to 2004, he worked as a visiting scholar the Ohio State University where he developed a technique to transform the potato late blight pathogen *Phytophthora infestans*, and studied functional roles of extracellular protease inhibitors proteins from *P. infestans*. At the end of 2004 he moved to the University of Basel, Switzerland, where he started a PhD program on *P. infestans* transformation and microarrays genechip analyses.

From January 2006 to February 2010, he worked as a PhD student (AIO) in the Laboratory of Plant Breeding at Wageningen University, The Netherlands. During his PhD, he studied the *Functional Genomics of Phytophthora infestans Effectors and Solanum Resistance Genes* under the supervision of Dr. Vivianne G.A.A. Vleeshouwers. This thesis presents the outcome of his four years of PhD research. Since April 2010, he obtained a Post-Doc position at The Sainsbury Laboratory (Norwich, United Kingdom), where he works on *Identification, isolation and characterisation of new sources of stem rust resistance in diploid relatives of cultivated wheat*. This project is supported by the Two Blades Foundation.

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List of Publications

Champouret N, Bouwmeester K, Rietman H, van der Lee T, Maliepaard C, Heupink A, van de Vondervoort PJ, Jacobsen E, Visser RG, van der Vossen EA, Govers F and Vleeshouwers VGAA (2009) *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. Mol Plant Microbe Interact 22:1535-45

Vleeshouwers VGAA, Rietman H, Krenek P, Champouret N, Young C, Oh SK, Wang M, Bouwmeester K, Vosman B, Visser RG, Jacobsen E, Govers F, Kamoun S, Van der Vossen EA (2008) Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. PLoS One **3(8)**:e2875

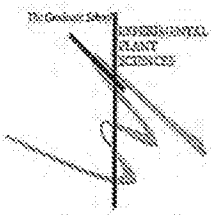
Champouret N, Verzaux E, Lokossou AA, Rietman H, Win J, Cano LM, Meijer DA, Budding D, Pelgrom K, Yang M, van der Vossen EAG, Jacobsen E, Visser RGF, Birch PRJ, Kamoun S, Vleeshouwers VGAA. Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species. PLoS Pathogen (Submitted).

Rietman H, Champouret N, Hein I, Niks R and Vleeshouwers VGAA. Plants and oomycetes, an intimate relationship: Co-evolutionary principles and impact on agricultural practice. CAB Abstract (Submitted)

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Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Nicolas Champouret
Date: 9 June 2008
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	date
➢ First presentation of your project	
Identification and characterization of Avr and associated R-genes: development of predicted durability resistance	Jan 2006
➢ Writing or executing a project proposal	
Identification and characterization of Avr and associated R-genes: development of predicted durability resistance	Oct 2006
➢ Writing a review or book chapter	
Plants and oomycetes, an intimate relationship: Co-evolutionary principles and impact on agricultural practice. CAB reviews. In press (2008).	Oct 2007
➢ MSc courses	
➢ Laboratory use of isotopes	
Safe handling with radioactive materials and sources	Nov 2006
Subtotal Start-up Phase	*13.0 credits**

2) Scientific Exposure	date
➢ EPS PhD student days	
EPS PhD student days (Wageningen)	Sep 19, 2006
EPS PhD student days (Wageningen)	Sep 13, 2007
1st Joint Retreat of the PhD Students in Experimental Plant Sciences (IMPRS, SDV and EPS)	Oct 02-05, 2006
➢ EPS theme symposia	
Theme 2: Interactions between Plants and Biotic Agents, University of Amsterdam	Feb 02, 2007
➢ NWO Lunteren days and other National Platforms	
NWO-ALW meeting Lunteren 2006: attendance	Apr 03-04, 2006
NWO-ALW meeting Lunteren 2007: attendance, poster	Apr 02-03, 2007
NWO-ALW meeting Lunteren 2008: attendance, presentation	Apr 07-08, 2008
NWO-ALW meeting Lunteren 2009: attendance	Apr 06-07, 2009
➢ Seminars (series), workshops and symposia	
Seminar Dr. Kurt H. Lamers:	Nov 08, 2006
Seminar Prof. Andrew Bent	Jan 18, 2007
Plant Breeding research day	Sep 27, 2007
➢ Seminar plus	
➢ International symposia and congresses	
Oomycete Molecular Genetics Network (Wageningen, The Netherlands)	May 05-06, 2006
Oomycete Molecular Genetics Network (Anaheim, USA)	Mar 18-20, 2007
24th Fungal Genetics Conference (Anaheim, USA)	Mar 20-25, 2007
XIII International Congress on MPMI (Sofia)	Jul 21-27, 2007
Oomycete Molecular Genetics Network (Warwick, UK)	May 06-08, 2008
SDL Meeting 2008, Cologne (Germany)	Oct 12-16, 2008
XIV International Congress on MPMI (Quebec, Canada)	Jul 19-23, 2009
➢ Presentations	
Oral presentation at international OMICS in Wageningen, 2006	May 05-06, 2006
Poster presentation at international OMICS in Wageningen, 2006	May 05-06, 2006
Poster presentation at Lunteren, OMICS and 24th FGC in Anaheim, 2007	Mar 19-25, 2007
Poster presentation at MPMI in Sorrento, 2007	Jul 21-27, 2007
Oral presentation at Plant Breeding research day, Wageningen, 2007	Sep 27, 2007
Oral presentation at Lunteren, OMICS in Bitozzo and Evrogen school, 2008	Apr, May, Jun, 2008
Poster presentation at SDL meeting in Cologne, 2008	Oct 12-16, 2008
Poster presentation at MPMI in Quebec, 2009	Jul 19-23, 2009
Oral presentation at TQS project (x6)	Apr 2008-Jun 2008
➢ IAB interview	Dec 2008
➢ Excursions	
Subtotal Scientific Exposure	*38.0 credits**

3) In-Depth Studies	date
➢ EPS courses or other PhD courses	
Somatic-related Signaling in plant development and defence towards systems biology	Jan 19-31, 2006
Gateway technology (hands-on)	Nov 20-24, 2006
Molecular Phylogenies: Reconstruction and Interpretation	Oct 15-19, 2007
Somatic-related: On the Evolution of Plant Pathogen Interactions: from Principles to Practice	Jan 28-29, 2008
➢ Journal club	
Literature discussion "Plant Breeding"	2006-2008
➢ Individual research training	
Collaboration on R3a gene shuffling, Scottish Crop Research Institute, Dundee, UK	Apr 18 - May 03, 2006
Subtotal In-Depth Studies	*10.0 credits**

4) Personal development	date
➢ Skill training courses	
Teaching and supervising Thesis students	Jan 15-19, 2006
Endnote 8 introduction and advance	Sep 28 and Oct 12, 2006
Basics Statistics	Dec 18-22, 2006
➢ Organization of PhD students days, course or conference	
Organization of the Lab-Trip of the Laboratory of Plant Breeding and PhD groups	Jun 01, 2006
Organization of the 1st Joint Retreat of the PhD Students in Experimental Plant Sciences	Oct 02-05, 2006
➢ Membership of Board, Committee or PhD council	
Member of EPS PhD student council	Nov 2007-Dec 2009
Subtotal Personal Development	*4.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	55.5

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits.

* A credit represents a normative study load of 28 hours of study.

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INHERITANCE OF PIGMENTED TUBER FLESH IN CULTIVATED DIPLOID POTATOES

H. De Jong[1]

Abstract

In a genetic study with cultivated diploid potatoes the distribution of anthocyanin to the tuber flesh was found to be controlled by a single dominant allele. The gene symbol *Pf* (pigmented flesh) is proposed. The gene *I*, which controls the distribution of pigment to the tuber skin, is necessary for the expression of *Pf* and is closely linked to the *Pf* locus. The *Pf* gene is a potentially useful genetic marker which does not appear to be associated with any deleterious traits and can be screened for without laboratory equipment.

Compendio

En un estudio genético con papas cultivadas diploides, se encontró que la distribución de antocianinas en la carne del tubérculo era contralada por un solo alelo dominante. Se propone para el gene correspondiente el símbolo *Pf* (pigmented flesh=carne pigmentada). Para la expresión del gene *Pf* se hace necesaria la presencia del gene *I*, el cual controla la distribución del pigmento a la piel del tubérculo y está estrechamente vinculado al locus *I*. El gene *Pf* es un marcador genético potencialmente útil que no parece estar asociado con ninguna característica negativa o perjudicial y puede ser detectado sin equipo de laboratorio.

Introduction

The distribution of anthocyanin pigments in potatoes can extend into various plant parts, including the tuber flesh (Figure 1). Primitive cultivars with pigmented flesh have been reported to be used for dyeing purposes (5).

The basic inheritance pattern for anthocyanin pigmentation in potatoes has been reviewed by Howard (7, 8). The loci *P* and *R* are responsible for the production of purple and red pigments, respectively. There are several genes that affect the distribution of pigments to various plant parts (2, 3, 4, 9). In order for a specific plant part to be pigmented, a dominant allele of the respective distribution gene for that part must be present in addition to a dominant allele of either one or both pigment production genes (*P* and *R*).

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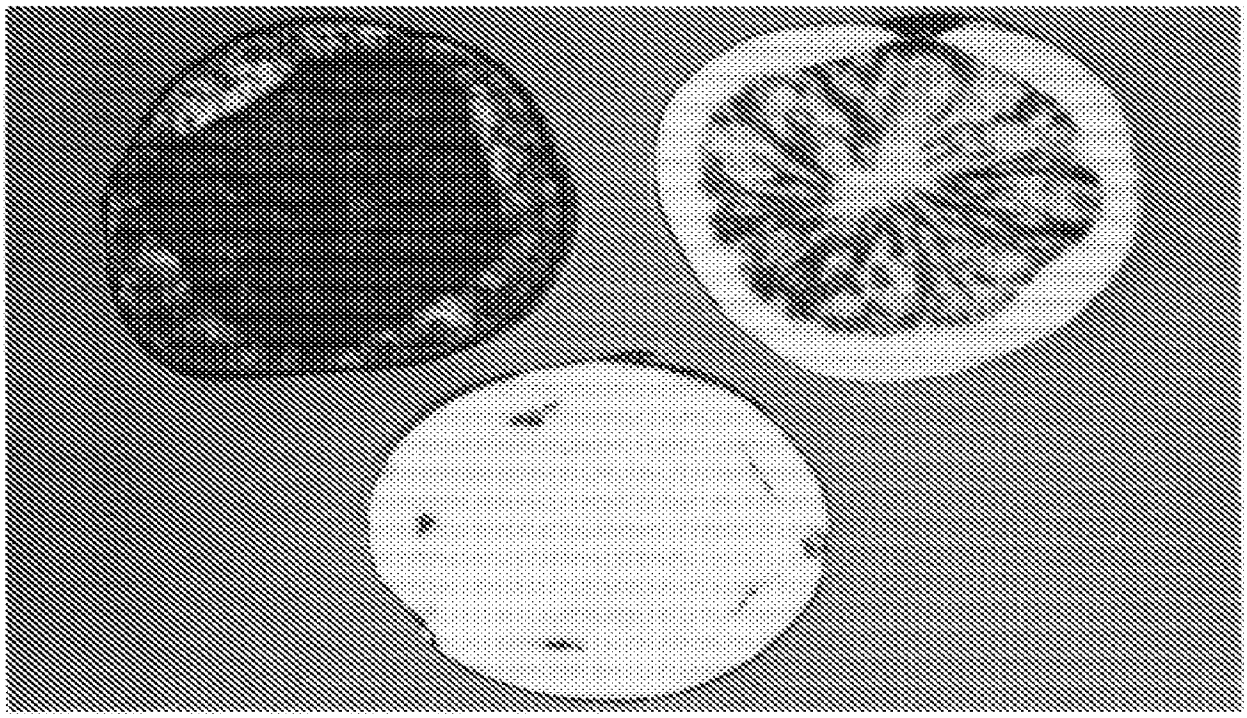


FIG. 1. Varying degrees of tuber flesh pigmentation.

Krantz (10) analyzed the progeny obtained by selfing Congo Black, a cultivar with pigmented flesh (presumed to be tetraploid), and concluded that the segregation ratio fit a pattern of a single dominant gene for flesh color. By selfing an unnamed cultivar with pigmented flesh, Mueller (11) obtained a ratio of approximately 9 pigmented: 7 non-pigmented and concluded that two dominant alleles (*C* and *I*) are required for the expression of pigmented flesh. He further postulated the presence of a third gene (*Z*), the dominant allele of which would act as an inhibitor of pigmented flesh. Austin, *et al.* (1) recently demonstrated the value of the pigmented flesh trait as a marker in a somatic fusion experiment.

Although the cultivated potato of North America (and most other parts of the world), *Solanum tuberosum* Group Tuberosum (tbr) is a tetraploid (2n=4x=48), the disomic inheritance pattern of cultivated diploid (2n=2x=24) potatoes makes them much more suitable for genetic analyses. This account describes the inheritance of the distribution of anthocyanin pigments in the tuber flesh of cultivated diploid potatoes.

Materials and Methods

Among the primitive diploid cultivars such as *S. tuberosum* Groups Phureja (phu) and Stenotomum (stn) clones with pigmented flesh (either red or purple) are quite common. The degree of tuber flesh pigmentation

can vary from just a slight pigmentation of the vascular ring to a complete pigmentation of the entire tuber (Figure 1). Thus far the diploid breeding project at the Agriculture Canada Research Station in Fredericton, N.B. has been primarily based on diploid hybrids between phu or stn and tbr haploids. Several clones were selected for various traits such as adaptation, tuber type, tuber size, yield, male and female fertility, and pigmented flesh. By analyzing segregating progenies, genotypes for several previously described marker genes can be assigned to parents in the diploid breeding project. The parents used in this study are described in Table 1.

Since the red pigment is often not as fully expressed as the purple or blue pigment, only clones with purple pigmented flesh were used as parents.

Results and Discussion

Progenies of 11 crosses were analyzed (Table 2). Since none of the white-skinned tubers had pigmented flesh, the former are not further considered in this discussion. Segregation for pigmented flesh occurred within that portion of each progeny that had pigmented tuber skin. This indicates that the dominant allele at the *I* locus, which controls the expression of anthocyanins in the tuber skin, is also necessary for the expression of anthocyanins in the tuber flesh. The segregation for pigmented flesh in families 1 to 4 fits a 1:1 ratio which suggests that this trait is controlled by a single dominant allele. The gene symbol *Pf,* already advanced by Rowe (personal communication, 1972) is proposed.

TABLE 1. — *Description of parents.*

	Pigmentation phenotype					
	Tuber				Genotype for some	
	Skin		Flesh		previously described	
Clone No.	+[1] or −	Color	+[1] or −	Color	pigmentation genes	
75-15	+	purple	+	purple	*Pp RR*	*II*
79-15	+	red	−		*pp* RR^{pw}	*Ii*
79-48	+	red	−		*pp Rr*	*II*
312-4	+	purple	+	purple	*Pp Rr*	*Ii*
320-1	+	red	−		*pp Rr*	*Ii* $B^{d}b$
7506-1	−		−		*pp rr*	*ii*
7539-1	+	purple	+	purple	*PP R-*	*Ii*
7553-1	+	purple	+	purple	*Pp Rr*	*Ii*
8132-2	−		−		*pp Rr*	*ii*
US-W7589.2*	−		−		*pp rr*	*ii*
US-W9234.3*	−		−		*pp R-*	*ii*

*Received from Inter-Regional Potato Introduction Station, Sturgeon Bay, Wisconsin.
[1]+ = pigmented.
− = non-pigmented.

TABLE 2. — *Segregation for pigmented flesh.*

Family	Parents		Observed		Expected: If *I-Pf* linked (assuming no c.-o)				Expected: If no linkage between *I* and *Pf*			Parental genotypes for *I* (see Table 1) and *Pf* (postulated)
	Female	Male	+[1]	−[1]	+	−	X^2	P	+	−	X^2	
1	79-48	7553-1	22	29	25.5	25.5	1.0	.30-.50	25.5	25.5	1.0	*II pfpf* x *Ii Pfpf*
2	75-15	7553-1	41	25	49.5	16.5	5.8	.05-.10	49.5	16.5	5.8	*II Pfpf Ii Pfpf*
3	75-15	7506-1	21	29	25	25	1.3	.20-.30	25	25	1.3	*II Pfpf ii pfpf*
4	75-15	8132-2	29	39	34	34	1.5	.20-.30	34	34	1.5	*II Pfpf ii pfpf*
5	312-4	320-1	44	14	38.7	19.3	2.2	.10-.20	29	29	6.5	*Ii Pfpf Ii pfpf*
6	79-15	7553-1	33	17	33.3	16.7	.01	.90-.95	25	25	5.5	*Ii pfpf Ii Pfpf*
7	7553-1	7506-1	23	3	26	0	.3	.50-.70	13	13	26.0	*Ii Pfpf ii pfpf*
8	7506-1	7553-1	24	0	24	0	0	1.0	12	12	24.0	*ii pfpf Ii Pfpf*
9	7539-1	US-W7589.2	51	0	51	0	0	1.0	25.5	25.5	51.0	*Ii Pfpf ii pfpf*
10	7539-1	7506-1	28	1	29	0	.03	.80-.90	14.5	14.5	29.0	*Ii Pfpf ii pfpf*
11	7539-1	US-W9234.3	26	0	26	0	0	1.0	13	13	26.0	*Ii Pfpf ii pfpf*
					Pooled	X^2	12.14	.30-.50				

[1] + = pigmented.
− = non-pigmented.

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In contrast to families 1 to 4 which did not segregate for tuber skin pigmentation, families 5 to 11 segregated for both tuber skin and flesh pigmentation. The segregation for tuber skin pigmentation is consistent with the parental genotypes as described in Table 1. The segregation ratios for pigmented flesh in these families are all highly significantly different from the 1:1 ratio which would be expected if *Pf* were segregating independently of *I*. For example, family 10 contained 29 plants with pigmented tuber skin. Without linkage between *I* and *Pf* the expected segregation for pigmented tuber flesh would be 14.5:14.5, which contrasts with the observed of 28:1. These results fit a hypothesis of close linkage between *I* and *Pf*, in this case in the coupling phase. The single plant with nonpigmented flesh represents progeny of a crossover gamete from the female parent. This confirms the observation by Krantz (10) that: "the factor for skin color was either absent or closely linked with flesh color." The data presented in Table 2 are internally consistent with the proposed genotypes of the parents for the *Pf* locus as well as for the joint segregation of *Pf* and *I*.

The degree to which the tuber flesh is pigmented in *Pf*-genotypes can be quite variable among, but usually not within, genotypes (Figure 1). Tuber flesh pigmentation can be considered as a product of a sequence of events in which genes for both pigment production (*P* and/or *R*), and distribution (*I*) play an essential role. Factors which affect one or more steps in this sequence may affect the amount and pattern of pigmentation of the tuber flesh.

The *Pf* gene does not appear to be a part of the hierarchy of multiple alleles at the *B* locus. One of the parents used in this study (320-1) has the B^d allele. This allele, in the presence of the pigment production gene *P*, gives rise to pigmented nodal bands and seeds with "embryo spot." According to Dodds and Long (3) B^d is the highest allele described thus far at this multiallelic locus; the dominance order is $B^d > B^c > B^b > B^a >$ b. Clone 320-1 does not have pigmented flesh. Furthermore, none of the parents with pigmented flesh (*P-I-Pf*) produced seeds with embryo spot. It can, therefore, be concluded that the *B* locus does not control pigmented flesh.

Genetic map distances between *Pf* and other known pigment distribution genes were not studied. For this purpose, several clones with a *PP* genotype are required. Since only one of the parents used in this study (7539-1) has the *PP* genotype, most progenies were segregating for purple and red pigments. Red genotypes (*ppR-*) frequently do not allow the pigment distribution alleles to be fully expressed which means that some *ppR-Pf-* genotypes could be misclassified as not having pigmented flesh. A parallel situation occurs with embryo spot; ppB^d-*R*- embryo spot is vague and difficult to classify (6). Mapping studies involving the *Pf* gene must await the development of this and other distribution genes in a more suitable genetic background. This will include good tuberization, flowering, male and female fertility and *PP* constitution. Improved genetic stocks could also be used to evaluate the

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model map proposed by Kessel and Rowe (9) for several other genes in this linkage group. A major gene for tuber shape also appears to be associated with this chromosome (2, 8), and thus further genetic studies of this linkage group are likely to result in an increased knowledge of the genetics of economically important traits.

The results of this study also emphasize the following points:

1. Genes controlling the production and/or distribution of anthocyanin pigments are excellent tools in potato genetics. For instance, whole plants can be studied and progenies segregating for most anthocyanin pigmentation genes can be scored visually in the field and/or greenhouse. Many potential genetic markers that affect the morphology and/or physiology of the potato plant frequently have deleterious effects (such as weak plants, sparse flowering, sterility, etc.) and are therefore often difficult to use in genetic studies. The anthocyanin pigmentation genes described thus far do not appear to be associated with any deleterious effect on either the growth and development or reproduction of the potato.
2. The linkage of *Pf* to *I* adds another gene to the same linkage group. All the anthocyanin pigment distribution genes described thus far are linked (2, 3, 4, 9). The significance of this was already discussed by Dodds and Long (3) when they discovered the linkage between *B*, *I* and *F* and reasoned that "The closeness of these loci evidently facilitates intergenic co-operation either in reaction or timing sequences of the development of anthocyanins and the production of pattern effects."

Further genetic studies with pigmentation genes in diploid potatoes should enhance our current limited knowledge of the genetics of this major world food crop.

Acknowledgment

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W. De Jong · A. Forsyth · D. Leister
C. Gebhardt · D. C. Baulcombe

A potato hypersensitive resistance gene against potato virus X maps to a resistance gene cluster on chromosome 5

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Abstract The dominant *Nb* gene of potato confers strain-specific hypersensitive resistance against potato virus X (PVX). A population segregating for *Nb* was screened for resistance by inoculating with PVX strain CP2, which is sensitive to *Nb*. Through a combination of bulked segregant analysis and selective restriction fragment amplification, several amplified fragment length polymorphism (AFLP) markers linked to *Nb* were identified. These were cloned and converted into dominant cleaved amplified polymorphic sequence (CAPS) markers. The segregation of these markers in a *Lycopersicon esculentum* × *L. pennellii* mapping population suggested that *Nb* is located on chromosome 5. This was confirmed by examining resistant and susceptible potato individuals with several tomato and potato chromosome-5-specific markers. *Nb* maps to a region of chromosome 5 where several other resistance genes–including *R1*, a resistance gene against *Phytophthora infestans*, *Gpa*, a locus that confers resistance against *Globodera pallida*, and *Rx2*, a gene that confers extreme resistance against PVX–have previously been identified.

Key words *Solanum tuberosum* · Resistance gene cluster · Potato virus X · *Phytophthora infestans* · AFLP

Introduction

Several dominant genes have been identified in potato that confer resistance against potato virus X in a strain-specific fashion. The *Nb* and *Nx* genes each confer hypersensitive resistance against PVX, while *Rx1*, and *Rx2* both confer extreme resistance against this virus (Cockerham 1970; Ritter et al. 1991). The panel of different interactions between PVX and potato resistance (R) genes constitutes an informative system to study the specificity of R gene-virus interactions.

Several PVX strains that differentiate potato R genes have been cloned and sequenced (Huisman et al. 1988; Skryabin et al. 1988; Orman et al 1990; Kavanagh et al 1992; Querci et al. 1993; Feigelstock et al. 1995). Through the construction of hybrids between virulent and avirulent strains, the viral determinants of interaction with *Nx* and *Rx1* have been identified (Santa Cruz and Baulcombe 1993; Goulden et al. 1993; Bendahmane et al. 1995). Both *Nx* and *Rx* appear to "recognize" the viral coat protein, although different features are perceived by each gene.

To more completely understand the basis of specificity in PVX-R gene interactions, it will be necessary to clone and characterize the corresponding R genes. Several R genes have recently been isolated and sequenced from other plants (reviewed in Jones 1996). Although the genes cloned to-date confer resistance against a variety of pathogens, including bacteria, fungi and viruses, clear sequence relationships are evident among these genes. Most of them code for a leucine-rich repeat sequence, an element that may be involved in signal perception, and some express kinase-like domains, possibly involved in signal transduction (Jones 1996). Many of these R genes were isolated by map-based means (Martin et al 1993; Mindrinos et al 1994; Grant et al. 1995; Song et al 1995; Dixon et al. 1996).

Genetic mapping studies have localized *Rx1* to the top arm of chromosome 12 and *Rx2* to the upper arm of chromosome 5 (Ritter et al. 1991). This information provides a useful starting point for the map-based cloning of these genes. Indeed, work is in progress to clone *Rx1* by positional means (A. Bendahmane, K. Kanyuka and D.B., personal communication). Less, however, is known about the chromosomal locations of the

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hypersensitive resistance genes *Nx* or *Nb*. Such information might reveal relationships between these genes and those conferring extreme resistance, or to genes conferring resistance to non-viral pathogens. Map information is also important if these R genes are to be cloned.

To determine where *Nb* is located, we examined progeny which resulted from selfing cv 'Pentland Ivory', a potato variety that carries *Nb*. Through the use of bulked segregant analysis (Michelmore et al. 1991) and AFLP technology (Vos et al. 1995), several markers linked to *Nb* maps to chromosome 5, close to the extreme resistance gene *Rx2*, as well as to *Gpa* (Kreike et al. 1994), a locus that provides resistance against *Globodera pallida*, and *R1* (Leonards-Schippers et al. 1992), a gene that confers hypersensitive resistance to *Phytophthora infestans*.

Materials and methods

Plant and viral materials

The virus-free potato cultivar 'Pentland Ivory' was obtained from the Agriculture and Fisheries Department, East Craigs, Edinburgh. Pentland Ivory carries the *Nb* resistance gene and was selfed to construct a population of 109 progeny segregating for *Nb*.

PVX strain CP, re-named CP2 in this paper to reflect that it is a group-2 strain as defined by Cockerham (1955), is unable to infect potato varieties that carry the *Nb* resistance gene (Moreira et al. 1980). Transcripts from a full-length cDNA alone (S. Santa-Cruz and D.B., unpublished results) of CP2 were synthesized in vitro to provide a genetically uniform source of inoculum, and were used to mechanically inoculate the cotyledons of young tomato seedlings. Two to three weeks after inoculation, virus particles were purified from infected tomato plants. Ten grams of symptom-expressing leaves were homogenized in 20 ml of 0.5 M sodium borate, pH 8.2, and then mixed with 30 ml of chloroform. The phases were separated by a 20-min centrifugation at 3500 g, and the aqueous supernatant was filtered through muslin into new tubes. For each 10 ml of supernatant, 5.25 ml of 20% (w/v) polyethylene glycol (PEG; $M_r = 8000$) was added. The solution was mixed, and left to stand for 1 h at 4°C. The virus was pelleted with a 10-min centrifugation at 13 200 g and then re-suspended in 10 ml of 4% (wv) NaCl. The resulting suspension was centrifuged again for 10 min at 13 200 g and the supernatant was then transferred to a new tube and mixed with 5.25 ml of 20% PEG. After 1 h 4°C, the virus was pelleted as above, re-suspended in 1 ml of 50 mM sodium borate, pH 8.2, and used immediately for potato inoculations.

DNA from F_2 progeny (41 individuals) of an *L. esculentum* × *L. pennellii* mapping cross was kindly supplied by C.M. Thomas (Sainsbury Laboratory, UK). The plants from which the DNA was isolated were originally provided by S. Tanksley (Cornell University, USA). Data for the segregation of over 1000 RFLP markers in these plants is available from the SolGenes Genome Database (http:\\probe.nalusda.gov:8000/cgi-bin/browse/solgenes).

A population of 461 diploid potato progeny segregating for *Phytophthora* resistance conferred by *R1* has previously been described (Meksem et al. 1995), as has a population of 44 diploid progeny segregating for *Rx2* (Ritter et al. 1991).

Screening of the mapping population for resistance against CP2

Stock plants of 109 Pentland Ivory S_1 progeny were maintained in the glasshouse (20°C). Two or more cuttings were taken from each stock plant, allowed to root, and then transferred to a controlled environment chamber (15°C, 85% humidity, 16-h photoperiod, light intensity of 100 $\mu E\,m^{-2}s^{-1}$) at least 1 week prior to inoculation. When the cuttings reached 10–20 cm in height, two leaves from each cutting were mechanically inoculated with CP2 virion preparation. Inoculated leaves were examined 8 days later for the presence of hypersensitive lesions. Three weeks after inoculation, systemic infection of non-inoculated leaves was tested by ELISA, using polyclonal PVX antisera [Agricultural Development and Advisory Service (ADAS), Harpenden] to coat microtitre plates, and monoclonal antibody MAC58 (Torrance et al. 1986) (supplied by ADAS) to detect the virus. Plants were considered resistant if they displayed hypersensitive lesions on inoculated leaves and virus could not be detected in non-inoculated leaves. Plants were considered susceptible if no local lesions were evident and virus could be detected in non-inoculated leaves.

Preparation of genomic DNA for AFLP analyses

To isolate genomic DNA, 5 g of potato leaves were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. This powder was transferred to a tube containing 30 ml of extraction buffer [100 mM Tris-Cl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 12.5% SDS, 8.3 mM NaOH, 0.38% w/v) sodium bisulphite, 0.38% (w/v) sodium diethyl-dithio-carbamate] pre-heated to 65°C. After ensuring that all the powder had been wetted, the suspension was placed at 65°C. After ensuring that all the powder had been wetted, the suspension was placed at 65°C for a further 30 min. Potassium acetate (9.3 ml of a 5-M solution) was added to the suspension, and the mixture was then placed on ice for 20 min. Particulate material was pelleted with a 10-min centrifugation at 2600 g and then the supernatant was filtered through muslin. Nucleic acids were precipitated by the addition of 0.7 vol of 2-propanol and pelleted immediately at 2600 g for 10 min. The pellet was washed with 70% ethanol and then dissolved in 2 ml of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). An equal volume of CTAB buffer (200 mM Tris-Cl, pH 8.0, 50 mM EDTA, 2 M NaCl, 2% CTAB) was added, and then the solution was mixed and incubated at 65°C for 15 min. The solution was extracted once with chloroform, the aqueous phase was transferred to a new tube, and 0.7 vol of 2-propanol were added. Nucleic acids were again pelleted with a 10-min centrifugation at 2600 g, washed with 70% ethanol, and re-suspended in 4 ml of 25 mM Tris-Cl pH 8.0, 10 mM EDTA. Genomic DNA was then further purified through CsCl density gradient centrifugation (Ausubel et al. 1987). Equivalent amounts of DNA from 20 resistant and 18 susceptible progeny, chosen at random, were bulked to create "resistant" and "susceptible" pools. Both pools were prepared for selective restriction fragment amplification as described by Thomas et al. (1995), using *Mse*I as the 'frequent' cutting enzyme and *Pst*I as the 'rare' cutting enzyme.

AFLP analyses

Selective restriction fragment amplification were performed, and the resulting products separated on 4.5% polyacrylamide gels, exactly as described by Thomas et al. (1995). Briefly, the resistant and susceptible pools were examined with 4 *Pst*I and 24 *Mse*I primers, giving a total of 96 different primer combinations. The *Pst*I primers were complementary to linkers that had been ligated to the *Pst*I sticky ends after digestion; in addition, the *Pst*I primers carried two extra nucleotides as their 3′ ends. Similarly, the *Mse*I primers were complementary to linkers ligated to *Mse*I sticky ends, and carried three extra nucleotides at their 3′ ends. The extra nucleotides at the 3′ ends of the *Pst*I and *Mse*I primers allowed for selective restriction fragment amplification. Side-by-side comparison of the amplified DNA of resistant and susceptible pools allowed easy detection of candidate AFLP markers linked in *cis* to *Nb*.

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Cloning of AFLP markers

Restriction fragments amplified specifically from the resistant pool represent sequences that may be linked in *cis* to *Nb*. Slices containing such fragments were excised from 4.5% acrylamide gels that had previously been dried on Whatmann 3 MM paper. The gel slices were re-hydrated in 1 ml of TE for 1 h at room temperature. The slices were then transferred to new tubes containing 200 µl of TE, crushed with a pipette tip, and left for another 2 h. After soaking, the crushed slice was pelleted with a brief centrifugation at 12000 g. One microliter of supernatant was then used as a template in a further polymerase chain reaction (PCR) amplification, using the same reaction conditions and the same pair of primers that had originally been used to amplify the restriction fragment from the resistant pool. These PCR products were than cloned directly into the vector pGEM-T (Promega). To confirm that a clone corresponded to the desired fragment, each clone was used as a template for a further amplification, using the same *Pst*I and *Mse*I primers and reaction conditions that had revealed the initial AFLP.

RFLP markers

The tomato chromosome-5 RFLP markers CT167 and TG432 (Tanksley et al. 1992) were kindly provided by S. Tanksley (Cornell University, USA). Map locations of potato chromosome-5 RFLP markers GP21, GP179 and GP186 (Leonards-Schippers et al. 1992) and conversions of GP21 and GP179 into PCR markers have previously been described (Meksem et al. 1995).

Conversion of AFLP and RFLP markers into cleaved amplified polymorphic sequence (CAPS) markers

The ends of AFLP and RFLP markers were sequenced with M13 forward and reverse primers. For all RFLP markers and large (>200 bp) AFLP markers, this sequence information was used to design oligonucleotide primers for direct PCR amplification of the RFLP or AFLP locus. For small AFLP markers (<200 bp), primers were first designed for a cycle of inverse PCR (I-PCR). Following the I-PCR protocol of Thomas et al. (1994), sequences flanking the small AFLP markers were cloned and sequenced, and this information was then used to design primers for direct PCR amplification of the AFLP locus. Undigested DNA from resistant and susceptible pools was amplified with forward and reverse primer pairs corresponding to each RFLP or AFLP locus. In all cases, amplification of resistant and susceptible pools resulted in products of identical size. To search for polymorphism between the PCR products of resistant and susceptible pools, the products of each were digested with a panel of restriction enzymes with four-base recognition specificities. If a polymorphism was detected after digestion, it was confirmed by examining individual members of the resistant and susceptible pools.

PCR-based screening of the mapping population

Initially, 109 Pentland Ivory S_1 progeny were screened for resistance and susceptibility. Some of the stock plants died or did not tuberise, leaving only 90 plants (68 resistant and 22 susceptible) for subsequent analysis. Template DNA for CAPS analysis (Konieczny and Ausubel 1993) was prepared from the 90 remaining plants by grinding a small leaf disc of each plant in a 1.5-ml tube containing 200 µl of 0.14 M sorbitol, 0.022 M EDTA, 0.8 M NaCl, 0.8% CTAB, 1% sodium n-lauroylsarcosine and 0.22 M Tris-Cl, pH 8. Chloroform (200 µl) was then added to each tube, the contents were mixed, and the emulsions were incubated at 65°C for 5 min. The tubes were centrifuged at 12000 g for 5 min, and then 100 µl of supernatant were transferred to a new tube containing 100 µl of isopropanol. DNA was allowed to precipitate for 15 min at room temperature, and then collected by a 15-min centrifugation at 12000 g. The resulting pellet was washed with 70% ethanol, air dried, and resuspended in 50 µl of water. Two microliters of this DNA were used as a template in amplification reactions. All CAPS analyses of individuals in the mapping population were conducted in 50-µl reaction volumes containing 10 mM Tris-Cl pH 8.3, 0.4 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 0.05% nonidet-P40 and 0.5 µM of each oligonucleotide primer. Primer sequences and details of thermal cycling conditions for each CAPS marker are given in Table 1.

Construction of map around *Nb* locus

To analyse the data from tetraploid Pentland Ivory S_1 progeny, we used the program MapMaker 2.0 (Lander et al. 1987), and assumed a negligible frequency of double reduction. Without double reduction, 50% of gametes from a simplex parent will carry *Nb*, which is the same proportion of gametes that would carry *Nb* if a heterozygous diploid parent had been used. Thus, the data were treated as if they had arisen from a diploid self (*Nbnb* × *Nbnb*).

Results

Development of a segregating population

The tetraploid potato cultivar 'Pentland Ivory' carries the *Nb* resistance gene. To construct a population segregating for *Nb*, Pentland Ivory was selfed. Two or

Table 1 PCR markers linked to *Nb*

Marker name	Forward and reverse primer sequences (5′ to 3′)	Cycling conditions (35 cycles)
GP186	TTCTATTACTCATGAAGGCTCTCC AATCATAATCACGACTCATTGTCC	94°C, 15 s; 55°C, 15 s; 72°C, 90 s
SPUD839	GACATGAGTTTTAGCAACAGTG AAGTAGCTGATATTGGGGATTC	94°C, 15 s; 55°C, 15 s; 72°C, 40 s
GP21	GGTTGGTGGCCTATTAGCCATGC AGTGAGCCAGCATAGCATTACTTG	94°C, 15 s; 60°C, 15 s; 72°C, 60 s
TG432	AAAGCAGTTGGATCTTCATCATCTC CGTTCAACACTGGTGCAATGAGAAG	94°C, 15 s; 55°C, 15 s; 72°C, 120 s
SPUD237	TTCCTGCTGATACTGACTAGAAAACC AGCCAAGGAAAAGCTAGCATCCAAG	94°C, 15 s; 55°C, 15 s; 72°C, 40 s
SPUD128	GTCAGCTCACGAATTCACATTCC GTGCAGACTCTGCCTAATCGACTC	94°C, 15 s; 50°C, 15 s; 72°C, 40 s

more cuttings from each of the S_1 progeny were screened for resistance to PVX by mechanically inoculating two leaflets of each cutting with strain CP2, which is sensitive to the *Nb* gene. By 8-days post-inoculation, 84 of the S_1 progeny had developed distinctive hypersensitive lesions on the inoculated leaves, characteristic of the *Nb*-mediated resistance response. The remaining 25 S_1 progeny did not develop lesions. Three weeks after inoculation, PVX could readily be detected by DAS-ELISA in the non-inoculated leaves of the 25 progeny which had not developed lesions, indicating that these plants were susceptible to PVX strain CP2. The observed 84:25 segregation ratio is a good fit to the 3:1 segregation ratio predicted if Pentland Ivory carries *Nb* in the simplex condition (i.e. genotype *Nbnbnbnb*). In a few cases, virus could be detected at levels comparable to susceptible plants in the non-inoculated leaves of plants which had developed local lesions. However, this was not reproducible for independent cuttings of any clone, and was always associated with the presence of hypersensitive lesions in the non-inoculated leaves, suggesting that PVX could occasionally escape the plant defences in the inoculated leaves, only to trigger the hypersensitive response again in the non-inoculated leaves.

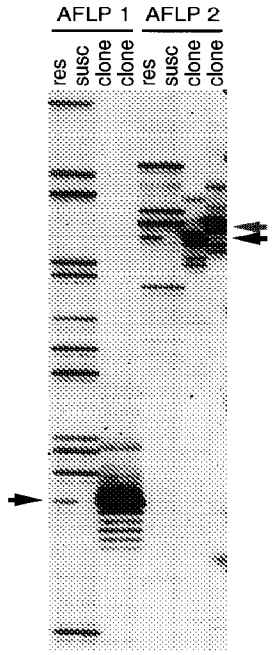


Fig. 1 Examples of two AFLP markers linked to *Nb*. Selected restriction fragments were amplified from pools of DNA from resistant (*res*) and susceptible (*susc*) S_1 progeny. *Black arrows* denote AFLPs, i.e. fragments specifically amplified from the resistant pool. Putative clones of each AFLP were amplified with the same primer combination to assess insert sizes. The *uppermost (grey) arrow* indicates an insert of inappropriate size.

Identification of markers linked to *Nb*

To identify markers linked to *Nb*, DNA from resistant and susceptible progeny was bulked to create resistant and susceptible pools. Each pool was then examined with 96 primer combinations, to identify AFLP markers linked to *Nb*. The primer combination each amplified approximately 100 fragments, so that about 9600 fragments were screened for linkage to *Nb*. Of the 96 primer combinations, seven revealed a total of eight fragments specific to the resistant pool. The AFLPs ranged in size from 40 to 400 bp; two representative AFLPs are shown in Fig. 1.

It is our long term goal to clone *Nb* by using closely linked DNA markers. In addition to identifying many AFLP markers linked to *Nb*, this process will require the identification of chromosomal recombination events near the *Nb* locus. To facilitate the screening of progeny for recombination events near *Nb*, several AFLP markers were cloned, and then converted into CAPS markers (Koniecany and Ausubel 1993).

To clone the AFLP markers the DNA was eluted from dried-down gels and re-amplified using the same primer combination that had originally been used to identify each polymorphic fragment. The resulting PCR products were then ligated into a plasmid vector. To assess whether the cloned fragments were of the same size as the initial AFLPs, the DNA of individual clones was amplified with the primer combination used to generate each AFLP, and the ensuing PCR products were compared with the original AFLP on a sequencing gel (Fig. 1). While most clones tested had inserts of the correct size, some did not (Fig. 1).

Cloned DNA corresponding to the size of the AFLP was sequenced. Based on the sequence, primers were designed for direct amplification of the corresponding locus if the AFLP was greater than 200 bp in length. Alternatively, if the AFLP was smaller than 200 bp in length, primers were designed for an intermediate step of inverse PCR (I-PCR). When I-PCR was employed, the I-PCR products were cloned and sequenced. Based on this sequence-data further primers were designed for direct amplification of the AFLP and its surrounding DNA.

After cloning and sequencing an AFLP marker it is not possible, without cloning and sequencing all alternative alleles, to determine what sequence polymorphism gave rise to the AFLP. Thus additional polymorphisms linked to resistance were sought by digesting the PCR products corresponding to each AFLP locus with a panel of restriction enzymes. For three of six cloned AFLP markers, this strategy led to the identification of DNA polymorphism linked to *Nb*. The CAPS marker SPUD237, illustrated in Fig. 2, was identified in this way, as were CAPS markers SPUD839 and SPUD128 (gel images not shown; primer sequences are given in Table 1).

Analysis of interspecific tomato cross suggests a chromosome-5 location

The genomes of potato and tomato show a remarkable conservation of gene order (Bonierbale et al. 1988;

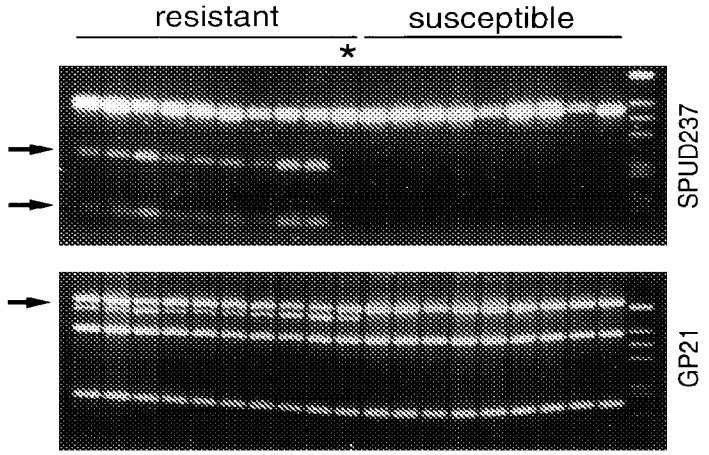


Fig. 2 Examples of two CAPS markers linked to *Nb*. DNA from ten resistant and nine susceptible S_1 progeny was PCR-amplified with SPUD237 forward and reverse primers, or GP21 forward and reverse primers (Table 1). After amplification, all PCR products were digested with *AluI*, separated on a 2% agarose gel, and visualized by staining with ethidium bromide. *Arrows* denote digestion products that are linked to resistance. The resistant plant marked with an *asterisk* carries a recombination event between *Nb* and SPUD237; this was the only recombinant identified between *Nb* and SPUD237 in a mapping population of 90 S_1 progeny

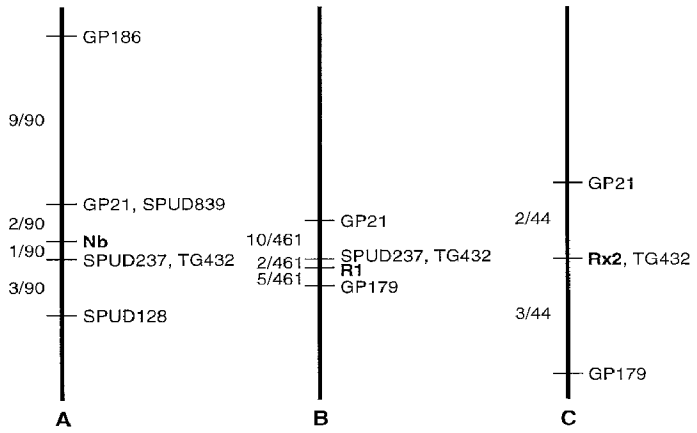


Fig. 3A–C Comparative map locations of *Nb*, *R1*, and *Rx2*. Genetic maps shown were obtained by mapping common markers in three different segregating populations (see Materials and methods). **A** Marker order around *Nb*. **B** Marker order around *R1*. **C** Marker order around *Rx2*. All markers shown are PCR-based. The SPUD markers were derived from AFLP markers linked to *Nb*, while GP21, GP179, GP186 and TG432 were derived from chromosome 5-specific RFLP markers of the same name. The number of recombination events/number of plants scored is shown to the left of each marker interval

Gebhardt et al. 1991). One high-density genetic map for solanaceous plants was obtained by scoring over 1000 RFLP markers in the F_2 progeny of an interspecific tomato cross (*L. esculentum* × *L. pennellii*; Tanksley et al. 1992). We found that two CAPS markers linked to *Nb*, SPUD237 and SPUD128, revealed a polymorphism between *L. esculentum* and *L. pennellii* (data not shown). The segregation of these markers in the tomato mapping population suggested a chromosome-5 location for *Nb*, as both SPUD237 and SPUD128 mapped to the upper arm of tomato chromosome 5, within 10 cM of tomato RFLP markers CT167 and TG432. This location was confirmed by RFLP analysis in potato with CT167 (data not shown) and was further confirmed by the conversion of potato and tomato chromosome-5 RFLP markers into CAPS markers that co-segregated with *Nb*. The co-segregation of *Nb* with CAPS marker GP21 is illustrated in Fig. 2. Similar data were obtained with tomato chromosome-5 marker TG432 and potato chromosome-5 marker GP186 (data not shown).

Construction of a genetic map around *Nb*

The segregation of six CAPS markers linked to *Nb* was monitored in a mapping population of 90 Pentland Ivory S_1 progeny. The data were analysed with MapMaker (Lander et al. 1987) and are summarized in Fig. 3 A. As the tomato data suggested, *Nb* maps to the upper arm of potato chromosome 5. *Nb* resides in an approximately 3-cM interval defined on the telomeric side by co-segregating markers GP21 and SPUD839 and, on the centromeric side, by co-segregating markers TG432 and SPUD237.

Nb is located in a resistance gene cluster

Three other resistance genes have previously been mapped to this region of chromosome 5. *R1*, a gene that confers hypersensitive race-specific resistance to *P. infestans*, was mapped 2.5 cM to the centromeric side of RFLP marker GP21 (Leonards-Schippers et al. 1992). *Rx2*, a gene that confers extreme resistance to most strains of PVX, was mapped 4.5 cm centromeric of GP21 (Ritter et al. 1991). *Gpa*, a locus that confers resistance to *G. pallida*, also maps to this area (Kreike et al. 1994). To further examine the relationship between *Nb* and *R1*, a population of 461 individuals segregating for *R1* was examined with the markers SPUD237 and TG432. *R1* was found to map just slightly centromeric to both of these markers (Fig. 3 B), confirming that *Nb* and *R1* are closely linked, and also demonstrating that *R1* and *Nb* are not allelic. In a smaller mapping population of 44 individuals segregating for *Rx2*, no recombinants were found between TG432 and *Rx2* (Fig. 3 C), showing that *Nb* and *Rx2* are also closely linked. Although *Nb*, *Rx2* and *R1* have all been mapped in different populations it is likely that these loci occupy linked positions in the potato genome.

Discussion

The potato resistance gene *Nb* maps to the upper arm of chromosome 5 in a region where several other resistance genes-*R1*, *Gpa*, *Rx2*-have previously been located. In addition to other examples in potato (Leister et al. 1996), clustering of disease resistance genes has also been observed in maize (McMullen and Simcox 1995), lettuce (Kesseli et al. 1993), and soybean (Kanazin et al. 1996; Yu et al. 1996). Clustering may reflect localized resistance gene duplication and subsequent divergence events, since the resistance genes sequenced to-date fall into a small number of related classes (reviewed in Jones 1996). Functionally, the clustering of resistance genes may facilitate coordination of defence against diverse pathogens. Further high-resolution characterization of chromosome 5 in the region of *Nb*, *R1*, *Gpa* and *Rx2* should provide useful insight into resistance gene organization and function.

It is particularly intriguing that *Nb*, a gene that confers hypersensitive resistance against some strains of PVX, maps so closely to *Rx2*, a gene that confers extreme resistance to most strains of the same virus. Our data do not exclude the possibility that *Nb* and *Rx2* are alleles, although higher-resolution map data would be needed to conclude this with any confidence. *Rx1* is known to "recognize" a feature of the viral coat protein (Goulden et al. 1993; Bendahmane et al. 1995), and *Rx2* apparently recognizes the same feature (Querci et al. 1995). The avirulence determinant of the *Nb*-PVX interaction has yet to be fully characterized, but it is not the PVX coat protein (W.D., A.F. and D.B., unpublished results).

Nb and the extreme PVX resistance gene X^i have previously been reported to be unlinked (Solomon 1985). This result apparently contradicts the finding reported here that *Nb* and *Rx2* are linked because it has been assumed that X^i and *Rx2* are homonyms. However, the nomenclature for the extreme resistance genes against PVX varies, and the relationships among the various genes is not always clear. In view of the close linkage between *Nb* and *Rx2*, it seems most likely that X^i is synonymous with *Rx1*, or conceivably a third *Rx* locus. The *Nb* gene studied by Solomon (1985) is very likely the same gene studied here, as marker SPUD237 is linked to both copies of the *Nb* gene present in cultivar 'Catriona' (W.D., A.F. and D.B., unpublished results), which was the source of *Nb* in the Solomon (1985) study.

The mapping of *Nb* was initiated with the long-term view of cloning this gene by "chromosome landing" (Tanksley et al. 1995). To generate a high-resolution map around the *Nb* locus will require both a large number of markers lined to *Nb* and a large number of recombination events around *Nb* to determine the relative order of all closely linked markers. With the advent of AFLP methodology (Vos et al. 1995) it is relatively straightforward to generate a large number of markers. Using just 96 primer combinations we identified eight markers linked to *Nb*. Several hundred more primer combinations are available, and so it should be possible to identify many more markers. The CAPS markers SPUD237 and GP21 (Fig. 2) flank *Nb*, and will be useful in identifying recombination events close to *Nb*. Some 700 further plants have already been screened with these markers, identifying additional recombinants in this interval (W.D and D.B., unpublished results) and providing further evidence that *Nb* maps between GP21 and SPUD237, rather than outside of this interval.

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ORIGINAL PAPER

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An allele of dihydroflavonol 4-reductase associated with the ability to produce red anthocyanin pigments in potato (*Solanum tuberosum* L.)

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Abstract The potato *R* locus is necessary for the production of red pelargonidin-based anthocyanin pigments in any tissue of the plant, including tuber skin and flower petals. The production of pelargonidins in plants requires the activity of dihydroflavonol 4-reductase (DFR) to catalyze the reduction of dihydrokaempferol into leucopelargonidin. To test the hypothesis that potato *R* encodes DFR, portions of both *dfr* alleles were sequenced from a diploid potato clone known to be heterozygous *Rr*. Sequence comparison revealed a polymorphic *Bam*HI restriction site. The presence or absence of this site was monitored in three diploid populations that segregated for *R*, as well as in a wide range of tetraploid breeding clones and cultivars, by amplifying a fragment of *dfr* and digesting the products with *Bam*HI. An identically sized *dfr* restriction fragment lacking the *Bam*HI site was present in all potato clones that produced red anthocyanin pigments, while the same fragment was absent in many potato clones with white tuber skin and flowers. An independent RFLP test using *Dra*I to detect sequence polymorphism was performed on a subset of the potato clones. This test also revealed *dfr*-derived bands that were present in all red-colored potatoes and absent in several white clones. The presence of shared restriction fragments in all red-colored potatoes provides strong evidence that *R* does indeed code for DFR. The data are also consistent with a 48 year-old hypothesis by Dodds and Long, that *R* was selected just once during the domestication of potato. A cDNA clone corresponding to the red allele of *dfr* was sequenced and compared to two other alleles. The red allele is predicted to encode a 382 amino acid protein that differs at ten amino acid positions from the gene products of the two alternative alleles. Several of these differences map in a region known to influence DFR substrate specificity in *Gerbera*.

Keywords Crop domestication · Secondary metabolism · Marker-assisted selection · Allele dosage

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Introduction

The skin of cultivated diploid and tetraploid potato (*Solanum* spp.) tubers can be colored either red, purple or white. This natural variation in color is controlled by three independent loci—*R*, *P* and *I* (reviewed in De Jong 1991). *R* and *P* are required for the production of red and purple anthocyanin pigments, respectively, anywhere in the potato plant, e.g. in tubers, flowers, sprouts or stems, while *I* is required for the tissue-specific expression of anthocyanins in tuber skin (Dodds and Long 1955, 1956). The tubers of wild potato species are only purple or white, which has led to the suggestion that the ability to produce red anthocyanin pigments was selected just once, and at the diploid level, during domestication (Dodds and Long 1955). *R*, *P* and *I* have been genetically mapped to chromosomes 2, 11 and 10, respectively (Van Eck et al. 1993, 1994).

Biochemical analyses have shown that the red anthocyanins in tuber skin and flesh are primarily derived from the anthocyanidin pelargonidin, while the purple pigments are derived from the related compound petunidin (Lewis et al. 1998; Naito et al. 1998; Rodriguez-Saona et al. 1998). Some pink-colored clones of the diploid species *Solanum phureja* and *Solanum stenotomum* have been shown to contain peonidin-derived pigments instead of

pelargonidin derivatives (Dodds and Long 1955). The enzyme dihydroflavonol 4-reductase (DFR) plays a necessary role in the synthesis of pelargonidins, peonidins and petunidins, although it catalyzes the reduction of slightly different substrates, dihydrokaempferol (DHK), dihydroquercitin (DHQ) and dihydromyricetin (DHM), respectively, in each case (Holton and Cornish 1995). The structure of DHK differs from DHQ and DHM in having one and two fewer hydroxyl groups, respectively, on the B phenyl ring. In some genera, including *Petunia* (Forkmann and Ruhnau 1987) and *Cymbidium* (Johnson et al. 1999), DFR is not able to efficiently reduce DHK although it is still able to reduce DHQ and DHM. In other genera, e.g. *Gerbera*, DFR can utilize DHK, DHQ and DHM as substrates (Johnson et al. 2001). The variation in DFR substrate specificity explains why red pelargonidin-pigmented flowers are observed naturally in *Gerbera* but not in *Petunia* (Johnson et al. 2001).

The *dfr* gene has previously been mapped in tomato to chromosome 2 (Bongue-Bartelsman et al. 1994). This chromosome shares extensive conservation of marker order with potato chromosome 2 (Tanksley et al. 1992). Because *R* also maps to this chromosome, and natural variation at the *R* locus is suggestive of variation in the substrate specificity of DFR, we wondered if *R* might correspond to *dfr*. To test this we followed the segregation of *dfr* alleles in several diploid populations that segregated for color, as well as in a wide range of tetraploid potato breeding clones and cultivars. We report here that all red-colored potato clones tested, but not all white potatoes, carry an allele of *dfr* that lacks a specific *Bam*HI restriction site. An independent RFLP test revealed that red-colored potatoes also share a common *dfr*-derived *Dra*I restriction fragment, which is absent in some white clones. These results suggest that *R* does indeed code for DFR, and are consistent with the hypothesis (Dodds and Long 1955) that the dominant *R* allele in modern potatoes results from the identification and subsequent selection of a single plant, most likely with red tuber skin, during crop domestication.

Materials and methods

Plant material

Ploidy and color characteristics of potato clones used in this study are summarized in Table 1. Several of the diploid clones have previously been assigned genotypes at two loci—*I* and *R*—that are together required for the synthesis of red anthocyanin pigments in tuber skin (De Jong 1991). These clones are 79-48 (genotype *II* RR^{pw}) (De Jong 1987), 320-02 (*Ii Rr*; unpublished), 07506-01 (*ii rr*) (De Jong 1987; De Jong and Burns 1983) and W5281.2 (*Ii RR*) (De Jong and Burns 1983). CIP590001.7 was described by Watanabe et al. (1994). Diploids W5281.2, W5337.3 and W5295.7 were developed at the University of Wisconsin. The NY clones listed in Table 1 were developed by the Cornell University potato breeding program. 320-02 was selfed to generate an S_1 population that segregates for tuber skin color. 79-48 was crossed as a female with 07506-01 to develop an F_1 population that segregates for flower color. 07248-02 was crossed as a female with 320-02 to generate an F_1 population that segregates for tuber skin color.

Allelic discrimination

In early experiments, template DNA for PCR amplification was isolated with a DNeasy plant mini kit (Qiagen). For some large-scale screening experiments potato DNA was isolated with the rapid miniprep method of Edwards et al. (1991). Both methods yielded acceptable results. PCR primers potDFR1 (*5′GGCTCTTG-GCTTGTCATGAG*) and potDFR2 (*5′AGCATTCCCCTGACT-GTTGG*) were designed to anneal to regions conserved between a tomato *dfr* cDNA sequence (GenBank accession Z18277) and a potato *dfr* EST sequence (accession BE923282). Based on comparison to the genomic DNA sequence of *Petunia dfrA* (accession X79723) the primers were anticipated to flank a region near the 5′ end of *dfr* that would include two small introns. The two primers were used to amplify genomic DNA of diploid 320-02 using the following thermal profile: 94°C for 2 min, followed by 35 cycles of 94°C, 15 s; 55°C, 15 s; and 72°C, 60 s. This and other amplifications below were carried out in a volume of 50 μl and contained 200 nM of each primer, 200 μM of dNTPs, 10 mM of Tris-Cl pH 8.3, 50 mM of KCl, 2.5 mM of $MgCl_2$, 0.05% of Nonidet-P40, 50–200 ng of template DNA and 2.5 units of *Taq* DNA polymerase. The resulting PCR products were ligated into vector pGEM-T following the manufacturer's (Promega) instructions. Eight independent clones were sequenced and aligned for comparison; this revealed a polymorphic *Bam*HI restriction site (see Results). To evaluate potato clones for this polymorphic site, PCR was performed with primers potDFR1 and potDFR2, and the products were then digested with *Bam*HI. Digested products were electrophoretically separated on a 2% agarose gel and visualized by staining with ethidium bromide.

RFLP analysis

DNA isolation and gel-blot analysis were essentially as described (Bonierbale et al. 1988). Genomic DNA was digested separately with *Dra*I or *Sca*I prior to blotting. An approximately 1.5 kb fragment of the tomato *dfr* gene, obtained by amplification with primers *5′CACTCTCCTCCGAAGACGAC* and *5′TCCATTGTCT-GCAGTGCTTC*, was used as a hybridization probe. Random hexamer labeling of the probe, hybridization and washing methods were as described previously (Feinberg and Vogelstein 1983). Filters were washed to a final stringency of 0.5×SSC at 65°C (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate).

cDNA library screening and cDNA sequencing

A cDNA library was constructed using a ZAP-cDNA synthesis kit (Stratagene) with mRNA isolated from pigmented flowers and stems of W5281.2. The tomato *dfr* probe described above was used for library screening. Positively hybridizing phage were plaque-purified and the inserts then excised to permit propagation in a pBlueScript SK(-) phagemid vector, following the manufacturer's instructions. The largest cDNA insert was completely sequenced on both strands. Partially sequenced (accession BG595686) potato cDNA clone cSTS11C14 was provided by S.D. Tanksley (Cornell University). This clone is derived from the potato cultivar Kennebec and was also completely sequenced on both strands.

Amplification and sequencing of dfr from genomic DNA

Primers to amplify the entire open reading frame of *dfr* alleles from W5281.2 and 07506-01 were designed based on the cDNA sequences obtained from W5281.2 and Kennebec, respectively. Primers DFRORF-F (*5′ CTTGAGGTTAAATTTTCTGACTC*) and DFRORF-R3 (*5′GCCTTTTTATTTTGTTAGGTTGTG*) were used to amplify *dfr* from W5281.2, while primers DFRORF-F and DFRORF-R (*5′ CCTTTTTATTTCATATGGTTGCG*) were used to amplify *dfr* from 07506–01 and the *dfr* allele linked to R^{pw} in 79-48. Thermal cycling parameters were 94°C for 2 min, followed by 35

Table 1 Characteristics of potato clones utilized in this study

Clone	Ploidy	Tuber skin color[a]	Flower color[a]	Sprout color[a]	*dfr* allele without *Bam*HI[b]	Genotype (where known)
07248-02	2*x*	White	White	ND[c]	No	
07506-01	2*x*	White	White	ND	No	*ii rr*
09479-05	2*x*	Red	Red	ND	Yes	
75-10	2*x*	White	Blue	ND	No	
79-48	2*x*	Red	Red	ND	Yes	*Ii* RR^{pw}
320-02	2*x*	Red	Red	ND	Yes	*Ii Rr*
11364-09	2*x*	White	Blue	ND	No	
11827-09	2*x*	Red	Red	ND	Yes	
AC Candy Cane	2*x*	Red	Red	ND	Yes	
CIP590001.7	2*x*	Red	Red	ND	Yes	
W5281.2	2*x*	Purple	Purple	Purple	Yes	*Ii RR*
W5295.7	2*x*	Red eyes	ND	Red	Yes	
W5337.3	2*x*	Red eyes	Red	ND	Yes	
Andover	4*x*	White	White	Purple	No	
Allegany	4*x*	White	Red	ND	Yes	
Atlantic	4*x*	White	Red	ND	Yes	
Banana	4*x*	White	Red	ND	Yes	
Chieftain	4*x*	Red	Red	ND	Yes	
Chippewa	4*x*	White	Red	Red	Yes	
Dakota Rose	4*x*	Red	Red	ND	Yes	
Dark Red Norland	4*x*	Red	Red	ND	Yes	
Elba	4*x*	White	Red	ND	Yes	
Eva	4*x*	White	White	White	No	
Genesee	4*x*	White	White	Purple	No	
Hampton	4*x*	White	White	Red	Yes	
Idarose	4*x*	Red	Red	ND	Yes	
Jacqueline Lee	4*x*	White	Red	Red	Yes	
Kanona	4*x*	White	Red	Red	Yes	
Katahdin	4*x*	White	Red	Red	Yes	
Kennebec	4*x*	White	White	White	No	
Keuka Gold	4*x*	White	White	Purple	No	
Lenape	4*x*	White	Blue	Purple	No	
Marcy	4*x*	White	White	Purple	No	
Monona	4*x*	White	White	Red	Yes	
Nordonna	4*x*	Red	Red	ND	Yes	
Pike	4*x*	White	White	Purple	Yes	
Reba	4*x*	White	White	Purple	No	
Red Companion	4*x*	Red	ND	ND	Yes	
Red Pearl	4*x*	Red	ND	ND	Yes	
Redsen	4*x*	Red	Red	Red	Yes	
Rideau	4*x*	Red	ND	ND	Yes	
Rosa	4*x*	Red eyes	Red	ND	Yes	
Salem	4*x*	White	White	Purple	Yes	
Serrana Inta	4*x*	White	Blue	Purple	No	
Snowden	4*x*	White	White	Purple	No	
Stirling	4*x*	White	White	Purple	No	
Superior	4*x*	White	Red	Red	Yes	
Yagana	4*x*	White	Red	Red	Yes	
Yukon Gold	4*x*	Red eyes	Red	Red	Yes	
NY97	4*x*	Red	Red	Red	Yes	
NY99	4*x*	White	Red	ND	Yes	
NY102	4*x*	White	White	Purple	No	
NY115	4*x*	White	White	Red	Yes	
NY118	4*x*	Red	Red	Red	Yes	
NY120	4*x*	White	White	Purple	Yes	
NY121	4*x*	White	White	White	No	
NY123	4*x*	White	Blue	Purple	No	
NY124	4*x*	White	White	Red	Yes	
NY125	4*x*	White	White	Purple	No	
NY126	4*x*	White	White	Purple	Yes	
NY129	4*x*	Red	Red	ND	Yes	
NYE11-45	4*x*	White	Red	Red	Yes	
NYL235-4	4*x*	White	Blue	Purple	No	
NYR6-4	4*x*	White	White	Purple	No	
NYR17-7	4*x*	White	Red	Red	Yes	
NYS48-6	4*x*	Red	White	ND	Yes	
NYT3-9	4*x*	White	White	Purple	No	
NYT15-1	4*x*	Red	Red	ND	Yes	

Table 1 (continued)

Clone	Ploidy	Tuber skin color[a]	Flower color[a]	Sprout color[a]	*dfr* allele without *Bam*HI[b]	Genotype (where known)
NYT15-3	4*x*	Red	Red	ND	Yes	
NYT17-2	4*x*	Red	Red	ND	Yes	
NYT28-1	4*x*	White	White	Purple	Yes	
NYU47-2	4*x*	White	White	Purple	No	
NYU75-1	4*x*	White	White	Purple	No	
NYU85-12	4*x*	White	White	Purple	No	
NYU100-87	4*x*	White	White	Purple	Yes	
NYU106-26	4*x*	White	White	Purple	Yes	
NYW2-8	4*x*	Red	ND	ND	Yes	
NYW2-19	4*x*	Red	ND	ND	Yes	
NYW2-24	4*x*	Red	ND	ND	Yes	
NYW2-37	4*x*	Red	ND	ND	Yes	
NYW2-42	4*x*	Red	ND	ND	Yes	
NYW2-112	4*x*	Red	ND	ND	Yes	
NYW2-123	4*x*	Red	ND	ND	Yes	
NYW2-127	4*x*	Red	ND	ND	Yes	
NYW3-10	4*x*	Red	ND	ND	Yes	
NYW3-31	4*x*	Red	ND	ND	Yes	
NYY4-1	4*x*	Red	ND	ND	Yes	

[a] Color was scored visually. "Red" denotes the presence of red pigment. For flowers and sprouts this included colors that ranged from light pink to deep red

[b] Presence or absence of the *dfr* allele lacking the polymorphic *Bam*HI restriction site (see text for details)

[c] Not determined

cycles of 94°C, 15 s; 50°C, 15 s; and 72°C, 150 s. Amplification products were ligated into pGEM-T. One full-length insert from each amplification was chosen at random and sequenced in its entirety on both strands.

Results

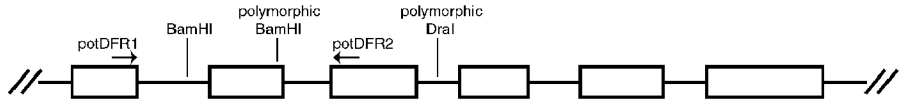
The red-skinned diploid potato clone 320-02 is heterozygous at the *R* locus (genotype *Rr*). To determine if 320-02 possesses two alleles of *dfr* a fragment of the gene was amplified with primers potDFR1 and potDFR2 (see Materials and methods). The resulting PCR products, approximately 530 base pairs (bp) in length, were cloned into a plasmid vector. Sequencing eight independent clones revealed the presence of two alleles, the sequences of which are shown in Fig. 1. Three of the clones matched allele 1 while the other five matched allele 2. The sequence data suggested that the two alleles would differ with regard to the presence or absence of a *Bam*HI restriction site (Fig. 1). The single C/T nucleotide polymorphism underlying the polymorphic *Bam*HI restriction site is in the wobble position of the 106th codon and is not predicted to change the amino acid sequence of DFR. To confirm the presence of this polymorphic site, 320-02 was again amplified with potDFR1 and potDFR2, and then uncloned products were digested with *Bam*HI (referred to hereafter as "the *Bam*HI assay"). The resulting banding pattern after electrophoretic separation on a 2% agarose gel was consistent with the presence of two alleles, with one allele yielding fragments of 370 and 162 bp and the other allele yielding fragments of 231, 158 and 144 bp (Fig. 2).

To determine if either allele could be related to red skin color 320-02 was selfed to yield an S_1 population of 56 individuals. Of these 39 had red skin and 17 had white skin. The *Bam*HI assay revealed that the 370-bp fragment was present in all 39 clones with red skin and absent in eight of the progeny with white skin (data not shown). The *R* locus in potato is necessary but not sufficient for the production of red anthocyanin pigments in tuber skin (Dodds and Long 1955, 1956). Such production also requires the action of a dominant allele at the *I* locus (Dodds and Long 1955, 1956). Because the 370-bp fragment was present in all red clones, *dfr* must be tightly linked to *R* or *I*. It is known that both *R* and *dfr* map to chromosome 2, while *I* maps to chromosome 10, so the linkage must be between *dfr* and *R*. The presence of the 370-bp fragment in nine of the white clones could be explained by assuming that some or all of these nine clones are homozygous recessive *ii*.

To further assess if *dfr* could be related to red skin color white-skinned diploid 07248-02, which does not exhibit the 370-bp fragment (Fig. 2), was crossed with 320-02 to yield an F_1 population of 199 individuals. Forty three of the progeny had red skin while the remaining 156 were white. The observed segregation ratio is consistent with 07248-02 having genotype *ii rr* and 320-02 having genotype *Ii Rr*. The 370-bp fragment was observed in all 43 red-skinned progeny as well as in 51 of the white-skinned clones (data not shown). The presence of the 370-bp fragment in all red-skinned progeny further established a tight genetic linkage between *dfr* and *R*.

A third population resulting from a cross between 79-48 (genotype *II* RR^{pw} *FF*) and 07506-*01 (ii rr ff*) was evaluated next. The *F* locus is required for the production

A



B

```
          ---- potDFR1 ------->
allele 1 GGCTCTTGGC TTGTCATGAG ACTCCTTGAA CGCGGTTATA ATGTCCATGC TACTGTTCGT  60
allele 2 GGCTCTTGGC TTGTCATGAG ACTCCTTGAA CGCGGTTATA ATGTCCATGC TACTGTTCGT  60

allele 1 GATCCTGgta cgttttcatt ttatttaaga gtttaacttc tatagat--- -tgtcaaata 116
allele 2 GATCCTGgta tgttttcgtt ttatttaa-- ------cttc tatagactgt ctgtcaaata 112

allele 1 atttataaac ccattaatca atctgtacaa agctgcattt tgaggatcca gagagatgtg 176
allele 2 atttataaac ccattaatca atccgtacaa agctgcattt tgaggatcca gagaagtgta 172

allele 1 tctcgttgt- ----atgcaa tgacccttat ataaagatgc atcaactttt gtagAGAACC 231
allele 2 tgacggtgct agaagttcaa tgacccttac ataaagatgc atgaactttt gtagAGAACC 232

allele 1 AGAAGAAGGT GAAACATCTG TTGGAACTGC CAAAAGCTGA TACAAACTTA ACGCTGTGGA 291
allele 1 AGAAGAAGGT GAAACATCTG TTGGAATTGC CAAAAGCTGA TACAAACTTA ACGCTGTGGA 292

allele 1 AAGCAGACTT GGCAGTGGAA GGAAGCTTTG ATGAAGCCAT TCAAGGCTGT CAAGGAGTAT 351
allele 2 AAGCAGACTT GGCAGTGGAA GGAAGCTTTG ATGAAGCCAT TCAAGGTTGT CAAGGAGTAT 352

allele 1 TTCATGTGGC TACACCGATG GATTTCGAGT CCAAGGACCC AGAGgtacta taaagtaaca 411
allele 2 TTCATGTGGC TACACCGATG GATTTCGAGT CCAAGGATCC AGAGgtacta taaagtaaca 412

allele 1 gagtaactca tcaatactaa tggacttctt acatttctgg atgattaata ctaacataac 471
allele 2 gagtaactca tcaatactta tggacttgtt acatttctgg atgattaata ctaacataac 472

allele 1 tcttatgaca ttgatatacg tgacagAACG AAGTAATTAA ACCAACAGTC AGGGGAATGC T 532
allele 2 tctcatgaca ttgatatacg tgacagAACG AAGTAATTAA ACCAACAGTC AGGGGAATGC T 533
                                        <----- potDFR2 ----- -
```

Fig. 1A,B Diagram of *dfr* gene organization and alignment of partial sequences of the two alleles of *dfr* present in diploid potato clone 320-02. **A** Schematic diagram of *dfr* gene organization. The *open boxes* represent coding sequence, which is divided among six exons. The locations where primers potDFR1 and potDFR2 anneal are shown, as are the locations of polymorphic *Bam*HI and *Dra*I restriction sites. The position of a conserved *Bam*HI restriction site is also shown. **B** Alignment of the sequence of two alleles of *dfr* present in diploid potato clone 320-02 between the annealing sites of primers potDFR1 and potDFR2. The regions where primers potDFR1 and potDFR2 anneal are highlighted with *hyphens and arrows*. Exon sequence is shown in *upper case type* while introns are denoted in *lower case*. Insertions or deletions of several nucleotides in one allele relative to the other are shown with *short dashes*. A *Bam*HI restriction site present in both alleles is underlined with a *thin line*. A polymorphic *Bam*HI site, present in allele 2 but absent in allele 1, is underlined with a *thick line*. The sequence of W5281.2 *dfr* in this region is identical to that shown for allele 1, except that the sequence of base 528 is G instead of A and base 531 is T instead of C

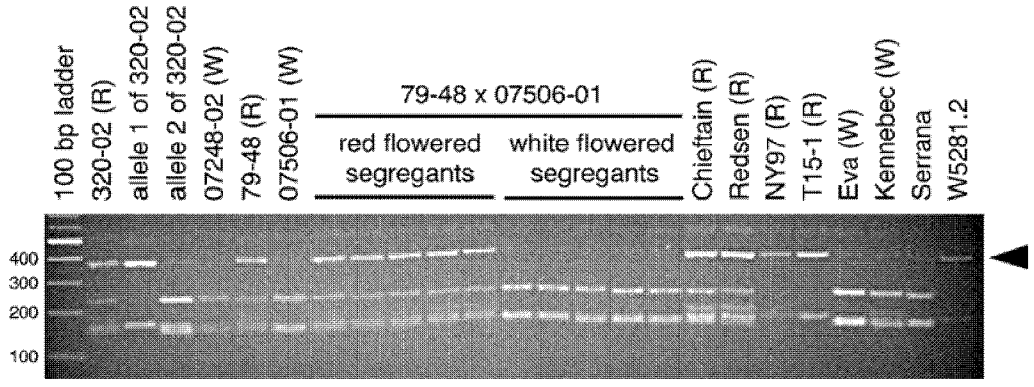


Fig. 2 Presence of a *dfr Bam*HI restriction fragment in red-colored potato clones and absence of this fragment in some white potato clones. Genomic DNA was amplified with primers potDFR1 and potDFR2, and then the products were digested with the restriction enzyme *Bam*HI. Digested products were separated on a 2% agarose gel, visualized by staining with ethidium bromide, and then photographed. DNA size markers, with the lengths of selected bands shown, were loaded in the *leftmost lane*. The allele 1 and allele 2 *lanes* show digestion products after amplification of cloned *dfr* fragments from 320-02 and correspond to allele 1 and allele 2 of Fig. 1. The following potato clones or cultivars have red tuber skin: 320-02, 79-48, Chieftain, Redsen, T15-1, NY97. Their names are followed by an R to indicate this. The following clones have white flowers and white tuber skin: 07248-02, 07506-01, Eva, Kennebec. Their names are followed by a W. Serrana has white tubers and pale-blue flowers. W5281.2 has purple-skinned tubers and is known to be homozygous *RR*. Digestion patterns for five red-flowered and five white-flowered progeny of 79-48×07506-01 are also shown

of anthocyanin pigments in potato flowers and is known to be tightly linked to *I* (Dodds and Long 1956). The phenotype of the R^{pw} allele has only been described to-date in several diploid species and is associated with the production of *p*ink-skinned tubers and *w*hite flowers (Dodds and Long 1955). The pink pigment in such tubers is derived from peonidin (a methylated derivative of cyanidin), not pelargonidin (Dodds and Long 1955). Of the 70 progeny evaluated, 28 had red flowers and red tubers while 42 had white flowers and pink tubers. 79-48 displayed a *Bam*HI fragment that migrated at the same position as the 370-bp fragment of 320-02, while 07506-01 did not exhibit a similar band (Fig. 2). Examination of their progeny revealed that this large fragment was present in all red-skinned/red-flowered clones and absent in all pink-skinned/white-flowered clones (some progeny are shown in Fig. 2). Thus, in the three segregating populations examined, the *Bam*HI assay revealed an apparently identical *dfr* restriction fragment in all progeny with red tuber skin.

Since 79-48 and 320-02 do not share any immediate ancestors yet nevertheless yield an indistinguishable 370-bp restriction fragment with the *Bam*HI assay, we wondered how widely distributed this fragment might be in other potato clones and whether the fragment would always be associated with the ability to produce red anthocyanin pigments. Accordingly a wide range of potato cultivars, breeding clones and diploid clones were evaluated with the *Bam*HI assay. All 46 cultivars and breeding clones tested with red skin, red flowers, and/or red sprouts (see Table 1) exhibited a *Bam*HI fragment indistinguishable in size from the 370-bp band of 320-02 (some clones are shown in Fig. 2). Two red-skinned breeding clones, NY97 and T15-1, appeared to be quadruplex for this allele since both clones yielded only two *Bam*HI fragments that co-migrated with the 370- and 162-bp fragments of 320-02 (Fig. 2). All six diploid clones tested with red skin or red tuber eyes also revealed fragments indistinguishable in size from the 370-bp fragments of 320-02 and 79-48 (data not shown).

Potato clones that do not produce any anthocyanin pigments in tubers, flowers or sprouts are relatively rare. The three such clones evaluated (Eva, Kennebec and NY121) did not display the 370-bp fragment (Fig. 2 and data not shown). Several potato clones (Lenape, Serrana, NY123, L235-4, 75-10 and 11364-09) appear, on the basis of their light blue flowers, to produce only blue petunidin-based pigments. If red pelargonidin-based pigments were also present the flowers would be expected to be purple. None of these six clones exhibited the large *Bam*HI fragment either (Fig. 2 and data not shown). Thus, similar to what was observed in the segregating diploid populations, an indistinguishable *dfr*-derived *Bam*HI restriction fragment was found in all potato cultivars, breeding clones and assorted diploid clones known to produce red pigments in their tuber skin, flowers and/or sprouts, while this fragment was absent in all potato clones tested that do not produce any red anthocyanins in the same tissues.

We also evaluated 21 white-skinned and white-flowered cultivars and breeding clones that produce purple pigments in tuber sprouts. The purple color suggests that all these plants possess a regulatory locus that directs expression of anthocyanin biosynthetic genes in sprouts, and that these plants can synthesize petunidin-based pigments; but because we have never observed blue-colored sprouts, even on plants with blue flowers, it was not possible to visually discern whether any of these plants also produced red pigment that might be masked by the production of purple pigment. Of the 21 clones, 14 did not display the 370-bp *Bam*HI fragment. The remaining seven (Pike, Salem, NY120, NY126, NYT28-1, NYU100-87 and NYU106-26) did reveal a large *Bam*HI fragment (data not shown).

To provide an independent test of whether unique or closely related alleles of *dfr* are associated with the ability to produce red pigments, we also subjected 17 potato clones to RFLP analysis, using a portion of the tomato *dfr* gene as a hybridization probe. As shown in Fig. 3 all tested clones known to produce red pigments displayed an identical RFLP marker allele approximately 3.6 kb in size after digestion with *Dra*I. This RFLP marker allele was absent in clones that do not produce red anthocyanins (Eva, Kennebec, Serrana, NY121, 07506-01) (Fig. 3). Probing *Sca*I-digested DNA blots yielded similar results; all clones known to produce red pigments shared a common RFLP band and this band was absent in clones free of red pigment (data not shown). Similar to the *Bam*HI data described above, conventional RFLP data also indicates that clones NY97 and T15-1 are quadruplex for the allele linked to red color as both clones displayed only the red-linked *Dra*I RFLP marker band (Fig. 3). Because the *Dra*I and *Sca*I tests assessed polymorphism distinct from the *Bam*HI assay, more than one sequence difference distinguishes the *dfr* allele(s) associated with red pigment production from all other *dfr* alleles.

The diploid clone W5281.2 is known to be homozygous *RR* (De Jong and Burns 1993). To provide a foundation for future comparison of *dfr* alleles a cDNA clone prepared from W5281.2 and a second cDNA clone obtained from the white-skinned cultivar Kennebec were completely sequenced (deposited in GenBank with accession numbers AY289921 and AY289922, respectively). These two cDNA clones as well as a third cDNA sequence from the purple-skinned cultivar Jashim (Jeon et al., unpublished; GenBank accession AF449422) are predicted to encode proteins of 382 amino acids (Fig. 4). The predicted W5281.2 gene product differs at ten amino acid positions from both Kennebec and Jashim (Fig. 4). The W5281.2 protein also differs at three additional positions from Kennebec, and at five additional positions from Jashim (Fig. 4). Of the three cDNA sequences only W5281.2 lacked the *Bam*HI restriction site highlighted in Figure 1. Curiously the W5281.2 cDNA sequence revealed two mismatches with the potDFR2 primer, albeit near the 5′ end of the primer, so that this primer is nevertheless able to participate in the amplification of this allele (Fig. 2). The sequence of

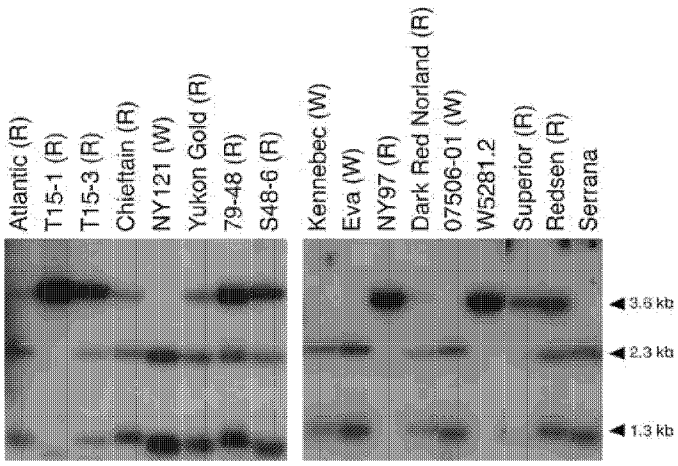


Fig. 3 RFLP analysis of potato *dfr*. Genomic DNA was digested with the restriction enzyme *Dra*I, separated by agarose-gel electrophoresis, blotted onto a nylon membrane, hybridized to a radioactively labeled tomato *dfr* gene fragment, and then the membrane was used to expose X-ray film. The samples loaded are shown above each lane. *The left and right hand panels* represent data from different experiments. Approximate sizes of restriction fragments are shown to the right. Clones denoted with an R have either red tubers or red flowers, or both. Clones denoted with a W have white tubers, flowers, and sprouts. Serrana has white tubers and blue flowers. W5281.2 has purple-skinned tubers and flowers and is known to be homozygous *RR*

"allele 1" shown in Fig. 1 was obtained from a PCR product where potDFR2 was one of the amplification primers. Thus if allele 1 shares these two nucleotide differences with W5281.2 they would not have been apparent when PCR products of allele 1 were sequenced.

Primers based on the 5′ and 3′ non-coding regions of W5281.2 and the Kennebec cDNA sequences were used to amplify genomic DNA between the translational start and stop codons of W5281.2 and 07506-01. Primers based on the Kennebec cDNA sequence were also used to amplify the *dfr* allele linked to R^{pw} in 79-48. The PCR products were cloned into a plasmid vector and then one randomly selected full-length clone from each genotype was completely sequenced (sequences not shown; genomic DNA sequence data for W5281.2 and 07506-01 have been deposited in GenBank with accession numbers AY289923 and AY289924, respectively). The sequence of W5281.2 in the region between the potDFR1 and potDFR2 annealing sites was found to be identical to the sequence of allele 1 shown in Fig. 1. The corresponding sequence of 07506-01 differs at several nucleotide positions from both allele 1 and allele 2 of Fig. 1, as well as from the Jashim cDNA sequence, and thus may represent a fourth allele of *dfr*. Interestingly, the genomic sequence of the *dfr* allele linked to R^{pw} in 79-48 was identical to the genomic sequence from 07506-01. Regions corresponding to the predicted exons of 07506-01 and 79-48 were identical to the cDNA sequence of Kennebec. A basis for the different *Dra*I RFLP patterns of Fig. 3 was found by comparing the genomic DNA sequence from W5281.2, 07506-01 and 79-48. W5281.2 contains no *Dra*I restriction sites within the gene, while 07506-01 and 79-48 carry a *Dra*I site approximately 350 bases downstream of the polymorphic *Bam*HI site, in the third intron (Fig. 1).

Discussion

The potato *R* locus encodes a basic factor required for the production of red pelargonidin-based anthocyanin pigments. To test the hypothesis that *R* encodes dihydroflavonol 4-reductase we monitored the segregation of

Fig. 4 Comparison of predicted potato DFR protein sequences. cDNA clones isolated from homozygous *RR* diploid W5281.2 (GenBank accession AY289921) and white-skinned tetraploid cultivar Kennebec (accession AY289922) were completely sequenced. Their predicted gene products and that from a *dfr* cDNA isolated from the potato cultivar Jashim (accession AF449422) are shown aligned. Each of the ten amino acid positions where W5281.2 DFR differs from both Kennebec and Jashim is highlighted in *black*. Other amino acid positions that are not conserved in these three alleles are highlighted in *gray*

```
W5281.2     MASEVHAVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL   60
Kennebec    MASEVHSVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL
Jashim      MASEVHAVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL

W5281.2     LELPKADTNL TLWKADLAVE GSFDEAIQGC QGVFHVATPM DFESKDPENE VIKPTVRGVL   120
Kennebec    LELPKADTNL TLWKADLAVE GSFDEAIQGC QGVFHVATPM DFESKDPENE VIKPTVRGML
Jashim      LELPKADTNL TLWKADLAVE GSFDEAISGC QGVFHVATPM DFESKDPENE VIRPTVRGML

W5281.2     SIIESCAKAN TVKRLVFTSS AGALDVQEDQ KLPCDETSWS DLDFIYAKKM TGWMYFVSKI   180
Kennebec    SIIESCAKAN TVKRLVFTSS AGTLDVQEDQ KLFYDETSWS DLDFIYAKKM TGWMYFVSKI
Jashim      SIIESCAKAK TVKRLVFTSS AGTLDVQEDQ KLFYDETSWS DLDFIYAKKM TGWMYFVSKI

W5281.2     LAEKAAMEEA KKNNIDLISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY   240
Kennebec    LAEKAAMEEA KKNNINFISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY
Jashim      LAEKAAMEEA KKNNINFISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY

W5281.2     VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP   300
Kennebec    VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP
Jashim      VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP

W5281.2     IVSFSSKKLM DMGFQFKYTL EDMYKGAIET CRQKQLLPFS TRSTADNGKD KEAIPIGTEN   360
Kennebec    IVSFSSKKLM DMGFQFKYTL EDMYKGAIET CRQKQLLPFS TRSSADNGKD KEAIPIGTEN
Jashim      IVSFSSKKLM DMGFQFKYTL EDMYKGAIET CRQKQLLPFH TRSTASNGKD KEAIPIGTEN

W5281.2     YSSGKENAPV ANCTGKFTNG EI  382
Kennebec    YSSGKENAPV ANCTGKFTNG EI
Jashim      YSSGKENAPV ANCTGKFTNG EI
```

dfr alleles in three diploid populations, as well as in a diverse set of diploid and tetraploid germplasm. In every case examined, potato clones known to produce red anthocyanins were found to contain an allele of *dfr* that lacks a *Bam*HI restriction site just upstream of the gene's second intron. The corresponding 370-bp *Bam*HI restriction fragment was absent in many white-skinned and white-flowered segregants. The notion that *R* might encode DFR was motivated by the precedent of DFR substrate specificity in *Petunia* (Forkmann and Ruhnau 1987) and *Cymbidium* (Johnson et al. 1999). In these two genera it is known that DFR can efficiently reduce dihydroquercitin (DHQ) en route to producing cyanidin-based pigments, and dihydromyricetin (DHM) en route to producing delphinidin-derived pigments, but not dihydrokaempferol (DHK), a step required to produce red pelargonidin-based pigments. DHK, DHQ and DHM are similar in structure, differing only in the presence of one or two hydroxyl groups on a phenyl ring that is not the site of enzymatic action. The *dfr* gene is known to be expressed in red tuber skin (Hung et al. 1997). Although the present study does not provide formal proof, given the combination of the biochemical precedent and the absolute co-segregation reported here, it appears very likely that *R* does code for DFR. It should be noted that because the polymorphic *Bam*HI and *Dra*I restriction sites described in this study are not expected to change the amino acid sequence of DFR, the functional polymorphism(s) responsible for red color almost certainly reside elsewhere within *dfr* or, if *R* does not encode DFR, in some other tightly linked gene.

Several alleles have previously been described at the *R* locus including the dominant *R*, associated with the ability to produce red pelargonidin pigments in tubers; R^{pw}, recessive to *R* but dominant to *r*, and associated with the production of peonidin-based pigments in tubers; and recessive *r*, associated with the absence of red tuber anthocyanins (Dodds and Long 1955; De Jong 1991). In the current study we found that the DNA sequences of *dfr* associated with *r* in 07506-01 and with R^{pw} in 79-48 were indistinguishable. This result was not a complete surprise. At the biochemical level the key difference between pelargonidin and peonidin synthesis is that peonidin requires flavonoid 3′-hydroxylase (F3′H) activity (Holton and Cornish 1995). F3′H catalyzes the hydroxylation of DHK, creating DHQ. The differential requirement for F3′H activity could be interpreted to indicate that the *R* locus codes for F3′H, although it is difficult to postulate natural variation in F3′H that would account for the spectrum of phenotypes and dominance relationships observed among *R*, R^{pw} and *r*. A plausible explanation can be devised, however, by postulating that the primary basis for the observed variation in tuber color results from the substrate specificity of DFR, and that superimposed on this is natural variation for tuber expression of F3′H. In this model the *dfr* allele present in all red-skinned potatoes encodes an isozyme that is able to utilize DHK as a substrate; this permits the synthesis of pelargonidins (the *R* phenotype). In genetic backgrounds that lack the red allele of *dfr* but do express F3′H, e.g. some clones of *S. phureja* and *S. stenotomum*, DHQ will be synthesized. Because DHQ can be utilized by all alleles of *dfr*, this permits the synthesis of pink peonidin-based anthocyanins (the R^{pw} phenotype). When neither DHK-competent DFR nor F3′H activities are present, no pink or red anthocyanins are produced (the *r* phenotype). Testing of this model will require extensive additional analyses at the biochemical level.

Domain swapping experiments between *Gerbera* and *Petunia* have shown that the ability of DFR to metabolize DHK resides in the first 170 amino acids (Johnson et al. 2001). Moreover, altering a single amino acid at position 134 has been shown to affect substrate specificity. The resulting *Gerbera* point mutant preferentially utilized DHK over DHQ and was no longer able to utilize DHM (Johnson et al. 2001). *Gerbera* amino acid position 134 corresponds to potato position 145. There are two differences in W5281.2 relative to Kennebec and Jashim near 145, at positions 143 and 154; both of these changes are in the third exon. If the red-linked *dfr* can metabolize DHK, these changes may be responsible.

Independent of whether or not *R* actually codes for DFR, from the perspective of potato domestication and current breeding, it is nevertheless intriguing that all red-colored potato cultivars appear to share an identical allele of this gene. Both the PCR/*Bam*HI test and conventional RFLP evaluation with *Dra*I and *Sca*I revealed indistinguishable bands in all red-colored clones tested. In the course of developing a TaqMan assay to measure dosage of the red-linked *dfr* allele we have also found that an oligonucleotide probe identical to bases 424 through 443 of allele 1 (numbering as in Fig. 1) specifically anneals to DNA from genotypes that generate the 370-bp PCR/*Bam*HI fragment (De Jong et al. 2003). Dodds and Long (1955) have previously noted that although pelargonidin-based pigments are present in many cultivated diploid and tetraploid potatoes they have never been reported in wild potato species. From this observation they hypothesized that the ability to produce red pigments in cultivated potatoes only arose once. They further postulated that a mutation conferring this ability was selected at the diploid level during domestication. Thus the gene controlling red color in modern cultivated tetraploids would be directly descended from the gene conferring color in the first domesticated red diploid. The data presented in the current study is consistent with the Dodds and Long scenario. One of the diploid clones evaluated, 79-48, has only two cultivated diploids, *S. phureja* and *S. stenotomum*, in its ancestry. As evident in Figs. 2 and 3, PCR/*Bam*HI and RFLP analyses revealed no differences between the red-linked allele of 79-48 and the allele shared by all modern red (tetraploid) cultivars.

The existence of a shared *dfr* allele also raises the question of whether alleles of any genes tightly linked to *dfr*, and present in the putative diploid ancestor, are also present in most or all modern red-colored potato cultivars. Because potato is clonally propagated it has undergone many fewer meioses during domestication than seed-

propagated crops like maize. Infrequent sexual reproduction could lead to multiple gene-containing linkage blocks that have never been separated by recombination. Further work to investigate whether there are indeed such linkage blocks in modern cultivars and, if so, determining how large such blocks are, would be of practical value to breeders in defining the genomic architecture of modern potato.

Potato breeding clones that are quadruplex for a desirable allele are valuable because they transmit the corresponding trait to all progeny. Two tetraploid potato clones evaluated in this study, NY97 and T15-1, are quadruplex for the red-linked allele of *dfr*, and thus may be of value in developing new red-skinned cultivars. The need for quadruplex *RRRR* and/or *IIII* clones is particularly acute when developing true potato seed (TPS) "varieties" that do not segregate for skin color. Several years ago while evaluating a breeding cross between NY97 and a North Dakota clone, ND2225-IR, we observed that all 184 progeny had red skin. When crossed with other clones both NY97 and ND2225-IR yield some white-skinned progeny. Taken together these observations suggest that one of these parents (NY97) is *RRRR* and the other (ND2225-IR) is *IIII*. The *Bam*HI assay described in this study provides a relatively simple means for identifying quadruplex *RRRR* clones. Should the absence of red color be desirable, the assay could also be used to identify *rrrr* individuals.

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Genetic analyses of root-knot nematode resistance in potato

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Jan Draaistra

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Chapter 1

General Introduction

Potato

The potato (Solanum tuberosum L. ssp. tuberosum) originates from the Andes in South America and has been distributed globally after its introduction in Europe around 1570 (Hawkes 1994). Potato is nowadays the most important non-cereal crop in the world and combines a high (potential) yield with a high nutritional value. The potential yield is often not reached due to biotic and non-biotic stresses (Oerke et al. 1994). Ranking fourth in food production with 311 million tons per year (http://apps.fao.org/default.jsp), genetic improvement of potato by means of plant breeding may contribute greatly to the world's food supply. Those improvements can be attained by resistance and tolerance to stress conditions and mechanical damage and by resistance and tolerance to the many diverse diseases and pests during growth and storage. Many resistance genes to the various diseases and pests are available in the broad gene pool of potato which contains some 235 species. Besides resistance, the Solanum species give access to various useful genes including tolerance to abiotic stress conditions like frost, heat and drought, and quality traits like enzymatic browning, dry matter content and protein composition and content.

The wild species used until now in potato breeding are mainly selected for their resistance characteristics. A total of 266 pathogens and pests are able to affect the potato (Mendoza 1987), of which the oomycete Phytophthora infestans is the most devastating. This disease causes enormous losses and has lead to the well-known Great Famine in Ireland in 1845 and 1846. As from that moment potato breeders tried to breed for resistance to late blight, using resistant wild potato species (Hawkes 1990). The introduction of wild potato species, for resistance breeding against P. infestans, caused most likely also the introduction of another important pest in Western Europe: the potato cyst nematodes Globodera rostochiensis and G. pallida. Soon after the detection of this pathogen, potato breeders looked for resistance in wild species. The first reported resistance to G. rostochiensis was the H1 gene from S. tuberosum ssp. andigena (Ellenby 1952). The successful introduction of resistance to G. rostochiensis is an example of the use of the genetic resources of potato. By extensive efforts in plant breeding, using wild species, a lot of cultivars resistant to Globodera spp. are nowadays available.

Most of the common cultivated potatoes are tetraploid with a somatic chromosome number of 2n=4x=48. The majority of the wild tuber-bearing Solanum species is however diploid (2n=2x=24). A breeding program of potato at the diploid level enables direct gene transfer from these wild diploid Solanum species to the tetraploid S. tuberosum. Such a diploid potato breeding program starts with the pollination of tetraploid potato cultivars with specific clones of S. phureja (2x), which results in the development of pseudogamous seeds (Hermsen & Verdenius 1973). The dihaploid clones derived from these crosses are the basis of the diploid breeding program. Subsequently, the dihaploid clones with the best agronomical performance are crossed with the wild diploid species. Crossing the clones with a high agronomic value with inferior but resistant wild species will mostly implicate a setback of performance of agronomic traits like tuber shape, length of stolons and eye depth. The resulting progeny of these crosses will also have an overall decreased quality, such as more discoloration after cooking or lower chipping quality. Several backcrosses will be necessary to return to an acceptable agronomic value. This backcrossing process is more efficient on the diploid level, due to the disomic

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inheritance vs tetrasomic inheritance on the tetraploid level, resulting in a larger phenotypic variation. Diploid breeding also implicates that a smaller progeny size and a lower number of backcrosses is required.

After cycles of breeding at the diploid level, the tetraploid level can be regained via 2n gametes. Of the two types of 2n-gametes the First Division Restitution (FDR) is preferred above the Second Division Restitution (SDR) because of its positive effect on yield (Hutten et al. 1994). Because most of the 2n-pollen is generated via FDR, and because the selection for 2n-pollen is less laborious than the selection for 2n-eggs, the recovery of the tetraploid level is preferred by means of 4x.2x crosses.

Besides the direct gene transfer and more efficient breeding, the use of diploid potatoes also has a major advantage in genetic studies. The polyploid nature of potato makes genetic research very complicated, especially when more genes are involved in the studied character.

Nematodes

Nematodes belong to a large phylum of worm-like animals of which a large part is plant pathogenic. The most economically important nematode species in relation to potato are the potato cyst nematode Globodera spp. and the root-knot nematode Meloidogyne spp.

Until the late 60s most of the diseases and pests, like P. infestans and potato cyst nematodes, were treated with pesticides. Due to environmental concerns the use of chemicals should be minimised as much as possible. The restriction of the use of chemicals in agriculture raised the need for resistant crops. Potato breeders have been successful in developing potato cultivars with resistance to Globodera spp. This success resulted in a drastic reduction of the use of nematicides. The drawback of the reduced use of chemical soil disinfection is the rising problem nowadays with other plant pathogenic nematodes, amongst others the Meloidogyne spp.

Three species of Meloidogyne are important in potato: the Northern root-knot nematode M. hapla, the Columbian root-knot nematode M. chitwoodi and the nematode M. fallax, described as biologically and morphologically different from M. chitwoodi (Karssen 1996). M. chitwoodi has been found in the following states of the USA: California, Colorado, Idaho, New Mexico, Nevada, Oregon, Utah, Washington and Texas (Szalanski et al. 2001); in Argentine, Mexico, Portugal, South Africa, The Netherlands, Belgium (summarised in Van der Beek 1997) and in Germany (Müller et al 1996). Populations of M. fallax are more frequently detected after the first description and molecular methods became available to identify the species (Zijlstra 1997). M. fallax is currently present in The Netherlands (Karssen 1996), France (Daher et al. 1996) and New Zealand (Marshall et al. 2001). World-wide the Northern root-knot nematode M. hapla is more widespread than M. chitwoodi and M. fallax.

The root-knot nematodes are like the Globodera spp. endoparasitic and mainly sedentary in the roots. These specialised plant parasites have a highly developed interaction with their hosts. The second-stage juvenile (J2) is the infective stage of the Meloidogyne nematode. Hatching from the eggs the J2 will penetrate the root tip and moves intracellularly towards the meristematic region. At the meristematic region the J2 turns and moves towards the vascular

cylinder where it induces a feeding site and becomes sedentary. The root-knot nematodes modify plant cells into several adjacent giant cells, where the potato cyst nematode induces syncytial feeding sites. Both feeding sites have intense metabolic activity and function as transfer cells for solutes. The root-knot nematodes also induce the plant to produce galls, surrounding them. Unlike the Globodera spp., where the female body develops into a cyst containing the eggs, the Meloidogyne spp. deposit their eggs in egg-masses, a gelatinous matrix attached to the female. The root-knot nematodes hibernate as eggs or as juveniles.

In contrast to the Globodera spp. whose host range is confined to several Solanum species, the Meloidogyne spp. are able to multiply on many diverse hosts. The host range of M. fallax and M. chitwoodi is rather similar, containing numerous dicotyledonous plants such as sugar beet, carrot, black salsify, lettuce, pea, alfalfa and tomato, but also cereals and grasses. Maize is the most prominent differential in defining the host range of the two species. Maize is a poor host to M. fallax and a good host to M. chitwoodi (van Meggelen et al. 1994). In M. chitwoodi two different races are recognised based on the reproduction on carrot and alfalfa. Thor alfalfa is a poor host to race 1 and a good host to race 2, where Red Cored Chantenay carrot is a good host to race 1 but a poor host to race 2 (Santo & Pinkerton 1985). This classification based on carrot and alfalfa is not always unambiguous, as race 2 sometimes produces egg masses on the non-host carrot (Van der Beek et al 1999). Based on the differences in reproduction on S. bulbocastanum carrying the R_{Mci} resistance gene, M. chitwoodi race 2 can be divided into two pathotypes, of which the virulent one was formerly designated as race 3 (Mojtahedi et al. 1994, 1998). The analysis of eight M. chitwoodi populations indicates that there is probably no race 2 present in The Netherlands (Van der Beek et al. 1999).

The host range of M. hapla is also very broad containing most dicotyledonous food crops, but this nematode species is not able to multiply on monocotyledons. Therefore, a crop rotation with monocotyledons is a feasible solution in order to control M. hapla. The inability of M. hapla to multiply on monocotyledons is most likely one of the reasons of its lower incidence on potato as compared with M. chitwoodi. Hilbrands Laboratorium voor Bodemziekten (pers. comm. J. Peltjes) observed less M. hapla infestations then M. chitwoodi infestations in the North-Eastern part of The Netherlands, an area with an intensive potato production. Another reason for the lower incidence of M. hapla on potato is the more rapid reproduction of M. chitwoodi at a lower soil temperature (O'Bannon & Santo 1984). In contrast to M. chitwoodi there are at present no host races described of M. hapla, but two cytological races of M. hapla are known: the most common meiotic parthenogenetic race A and the obligate mitotic parthenogenetic race B. Both cytological races are found in The Netherlands.

The symptoms caused by an infection of M. chitwoodi and M. fallax are similar. Tubers of infected plants show varying degrees of galling and the infestations give the tuber skin a rough appearance. Galls in freshly harvested potato tubers are translucent and difficult to detect, but after a few months the egg sacs turn brown and can be seen as brown spots in the flesh. The dark necrotic spots in the tuber flesh, the galled tubers and the rough skin turn the infected potato an unmarketable product. These typical symptoms are absent in tubers infected with M. hapla. The symptoms caused by this species in potato are restricted to losses in yield. A yield loss of 70% has been reported at a high level of infection with M. hapla (MacGuidwin & Rousse

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1990). In addition, the multiplication of M. hapla during the growth of a susceptible potato will result in higher residual population densities and can lead to severe damage to the next crop in the rotation, like carrot, onion and black salsify.

The wide host range of M. chitwoodi and M. fallax makes crop rotation not a feasible solution to decrease the nematode population and to minimise yield losses. In order to minimise the chemical input in agriculture, the development of resistant plants offers an environmental friendly solution to nematode infestations. The current lack of resistance in the cultivated potato forces the breeder to using wild Solanum species. Resistance to M. chitwoodi and M. hapla was detected in S. bulbocastanum and S. hougassii (Brown et al. 1989, 1991). Because S. bulbocastanum could not be crossed with cultivated potato, somatic hybridisation was used between this wild diploid Mexican species and a tetraploid S. tuberosum in order to introgress the resistance in cultivated potato (Austin et al. 1993). Backcrosses of the somatic hybrid resulted in resistant clones, and subsequently genetic analysis led to the mapping of the monogenic resistance gene R_{Mc1} on chromosome 11 (Brown et al 1995, 1996).

The introgression of the resistance present in the hexaploid Mexican wild species S. hougasii was easier because this species was crossable with S. tuberosum. It is suggested that this wild species carries independent loci involved in resistance to both race 1 and race 2 of M. chitwoodi (Brown et al. 1999). Additionally a broad set of other wild Solanum species has been tested for resistance to M. chitwoodi, M. fallax and M. hapla (Janssen et al. 1996a, 1997b). Populations produced from intraspecific crosses of several wild Solanum species were tested to dissect the genetics of the resistance (Janssen et al. 1997a). First attempts were also made to introgress resistance into cultivated potato using the wild species S. bulbocastanum, S. cardiophyllum, S. brachistotrichum, S. stoloniferum, S. fendleri and S. hougasii, of which only the latter three crossings were successful (Janssen et al. 1997c).

A major aspect that could influence the durability of the resistance is the presence and allele frequency of (a-)virulent factors in the pathogen. Therefore, the strategy to exploit resistance genes against the root-knot nematodes should dependent on the variation in virulence in natural populations. Virulent individuals are able to reproduce on resistant host plants. Knowledge of the virulence factors in the nematode population is also helpful in the search for additional resistance genes. The current lack of resistance in cultivated potato explains why only a minor proportion of the variation in virulence of M. chitwoodi, M. fallax and M. hapla to the tested potato cultivars could be described (Van der Beek et al. 1998a). The presumed genetic factors causing the variation should, therefore, be very small. There was, however, a significant difference in aggressiveness between the tested nematode populations. Other studies using resistant wild Solanum spp. detected significant variation in virulence between and within the Meloidogyne spp.. M. hapla more often showed a difference in virulence as compared with M. chitwoodi and M. fallax. Although the virulence of the latter two species is very similar on the different tested Solanum spp. there is a difference in reproduction on S. chacoense. This wild Solanum species is a good host for M. chitwoodi, but rather resistant to M. fallax (Van der Beek et al. 1998b, Janssen et al. 1997b).

Van der Beek et al. (1998b) detected little variation in virulence to S. chacoense and S. stoloniferum among the tested M. chitwoodi populations. The variation in virulence within the M.

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chitwoodi populations to the resistance in S. bulbocastanum has led to the nomination of race 3, later described as a virulent race 2 population (Motjahedi et al. 1994, 1998). Selection of M. chitwoodi on a resistant S. fendleri clone led to a virulent population of M. chitwoodi race 1 population (Janssen et al. 1998). Until now the amount of virulence factors present in M. chitwoodi is unclear and should be specified by testing a wide range of wild Solanum species and using the molecular data of the M. chitwoodi populations. Molecular analyses with two-dimensional gel electrophoresis (2-DGE) of different populations of M. chitwoodi, including the races 1 and 2 from the USA and the virulent race 2 population, did not show clear genetic differentiation between the populations (Van der Beek et al. 1997).

The results of 2-DGE of M. hapla show that the tested populations are rather heterogeneous as compared with M. fallax and M. chitwoodi (Van der Beek et al. 1997). Considerable variation in virulence within M. hapla populations was found to three wild Solanum species: S. chacoense, S. hougasii and S. sparsipilum (Van der Beek et al. 1998b). Although the genetic variation between the two cytological races of M. hapla was significant, no association could be found between the virulence and these races. Based on the estimated interaction between the M. hapla populations and the three Solanum spp. Van der Beek et al. (1998b) suggested that approximately five different virulence factors are present in the M. hapla populations.

Mapping of resistance genes

The developments and improvements in DNA marker technology has led to an increased understanding of genetics and has opened the possibility for an accurate connection of the genetic information with the phenotype. The diversity of molecular DNA markers available nowadays and the ongoing development in genomics offers the potato breeder a valuable tool for selection that should lead to an improvement of selection efficiency. The basic principle of marker assisted selection (MAS) is the selection of characters with the help of easily detectable phenotypes or DNA markers. Such a marker tightly linked to the gene of interest, will most likely be passed together with the character of interest to the next generation.

The advantages of MAS over phenotypic screening are evident in the case of characters expressed late in plant development or to characters that inherit recessively. The introgression of genes from wild species can be accelerated using MAS in the successive backcrosses. MAS can be used for the positive selection of the gene of interest and the recipient genome, and negative selection of the donor genome. MAS allows the selection for resistance without the need to use a resistance test. This is relevant when the pathogen is a quarantine organism and special precautionary measurements should be taken for the phenotypic screening.

Molecular markers can also be used to select for complex traits, such as yield or starch content. These complex traits are usually of a quantitative nature and strongly effected by the environment. By dissection of the trait into Mendelian loci, selection can be carried out at these so called quantitative trait loci (QTL loci), without looking at the phenotype and thus without the effects of the environment on the genotype. Especially in case of traits with a low heritability the use of molecular markers is a requisite.

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Marker technologies

With the development of the AFLP technology (Vos et al. 1995) a powerful molecular marker technique was added to the existing techniques as RFLP, RAPD and SSR. The AFLP marker technology resulted in an easily achieved and high amount of markers and accelerated the genetic research. The large numbers of markers allowed the construction of a marker saturated linkage map of potato (Van Os 2005). Many genetic studies in potato make use of the AFLP technology nowadays, but also SSR and CAPS are used. The latter two locus specific marker technologies make use of sequence information for the construction of primers. One of the advantages of AFLP markers is that no sequence information is necessary. The AFLP technique is, however, too expensive to be used for a high throughput MAS. Therefore, the AFLP markers need to be transformed into less expensive markers. Recently, a new technique was published that simplifies the transformation of AFLP markers into CAPS or SNP markers (Brugmans et al. 2003). The most ideal marker for a breeder is a SNP based on the sequence information of the alleles of the gene of interest. The transformation of AFLP markers into SNPs makes the MAS more cost-effective and opens the opportunity to multiplex different traits for MAS.

Genetic map

The DNA markers as described above, will behave as genes and inherit in a Mendelian way. Co-segregation of the molecular markers allows the construction of a genetic map. In order to handle the huge amount of marker data, computer programmes are developed to estimate the recombination frequency between the markers and to order the markers. In this way the first genetic map of potato was constructed using 134 RFLP and isozyme markers (Bonierbale et al. 1988). Later the first genetic map of potato using AFLP markers was published, by adding 264 AFLP markers to 217 RFLP, isozyme and morphological trait loci (Van Eck et al. 1995). Recently an ultra high density map of a diploid potato population was constructed containing about 10,000 markers (http://potatodbase.dpw.wur.nl/UHDdata.html, van Os 2005). A large amount of these AFLP markers has been shown to be chromosome specific and can be detected by co-migration on the gel combined with a similar mapping position (Rouppe van der Voort et al. 1997a).

Molecular markers have proven to be useful in map-based gene cloning projects. After the construction of a high resolution map surrounding the gene of interest, the gene can be located in a BAC library by chromosome walking. For example the R1 gene conferring resistance to *P. infestans* was mapped for the first time in 1992 (Leonards-Schippers et al. 1992), later this gene was fine-mapped (Meksem et al. 1995) and finally cloned (Ballvora et al. 2002). Other cloned resistance genes in potato are the Gpa2 gene conferring resistance to *G. pallida* (Van der Vossen et al. 2000), the Rx gene conferring resistance to Potato virus X (Bendahmane et al. 1999), the genes RB (Song et al. 2003), Rpi-blb1 (Van der Vossen et al. 2003) and R3a (Huang et al. 2005) conferring resistance to late blight.

Mapping strategy

Mapping strategy is depending on the inheritance of the trait. In case of a monogenic inheritance with a distinct segregation, the Bulked Segregant Analysis (BSA) (Michelmore et al. 1991) approach is a very effective way for the detection of linked markers, especially with the high amount of AFLP markers per primer combination found in potato. Only the putative linked markers found with the BSA will be tested on all individual genotypes of the mapping population. This will result in a small piece of the genetic map including the resistance gene. The BSA approach has shown its value in mapping studies using diploid populations (a.o. Hämäläinen et al. 1998, Tommiska et al. 1998) as well as tetraploid populations (a.o. Li et al. 1998). However, many important characters exhibit a continuous variation and are often controlled by multiple genes. Usually these characters are influenced by environmental factors, hampering a clear insight into the genetics of the character. In order to locate a QTL the construction of a genetic map of the complete genome is inevitable. The known mapping position of many AFLP markers in potato simplifies the construction of these maps. Many resistance genes, QTLs as well as major genes (M) have been localised in different published maps. In Table 1 resistance genes mapped so far are listed.

Table 1: Overview of resistance genes localised on the genetic map of potato.
Type M = monogenic qualitative gene; type QTL = quantitative gene.

Nematode resistance Name	nematode	type	source	chromosome	reference
H1	G. rostochiensis Ro1	M	adg CPC 16903	5	Pineda et al. 1993; Gebhardt et al. 1993
H3	G. pallida	QTL	adg CPC27754	5	Bradshaw et al., 1998
Gro1.1	G. rostochiensis Ro1	M	s.tvm (VT) 62-33-3	5	Jacobs et al. 1996
Gro1	G. rostochiensis Ro1 Ro5	M	spg H80.696/47	5	Barone et al. 1990
Gro1.2	G. rostochiensis Ro1	QTL	spg10	5	Kreike et al. 1993
Gro1.3	G. rostochiensis Ro1	QTL	spg11	5	Kreike et al. 1993
Gro1.4	G. rostochiensis Ro1	QTL	spg3	5	Kreike et al. 1996
Grp1	G. spp. Ro5, Pa2, Pa3	QTL	78-37785	5	Rouppe van der Voort et al. 1998b
Gpa2	G. pallida	M	adg CPC 167312	5	Rouppe van der Voort et al. 1997b
Gpa3	G. pallida Pa3	M	tar11	5	Wolters et al. 1998
Gpa4	G. pallida Pa2, Pa3	QTL	spt5	5	Wolters et al. 1998
sGpaV6.5	G. pallida Pa2, Pa3	QTL	spt5	5	Caromel et al. 2005
sGpaXI6.5	G. pallida Pa2, Pa3	QTL	spt11	5	Caromel et al. 2005
Gpa	G. pallida Pa2, Pa3	QTL	spg5	5	Kreike et al. 1994
Gpa	G. pallida Pa2, Pa3	QTL	tbr4	5	Bradshaw et al. 1998
Gpa5	G. pallida Pa2, Pa3	QTL	3704-765	5	Rouppe van der Voort et al. 2000
Gpa6	G. pallida Pa3	QTL	3704-769	5	Rouppe van der Voort et al. 2000
GpaM1	G. pallida	QTL	spg5	5	Caromel et al. 2003
GpaM2	G. pallida	QTL	spg6	5	Caromel et al. 2003
GpaM3	G. pallida	QTL	spg12	5	Caromel et al. 2003
Rmc1	M. chitwoodi	M	blb11	5	Brown et al. 1996; Rouppe van der Voort et al.1999

Fungus resistance Name	fungus	type	source	chromosome	reference
Sen1	S. endobioticum	M	tbr	11	Hehl et al. 1999

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Oomycete resistance Nameoomycete	type	source	chromosome	reference
R1P. infestans	M	dms	5	Leonards-Schippers et al. 1992
R2P. infestans	M	dms	4	Li et al. 1998
R3aP. infestans	M		11	Huang 2005
R3bP. infestans	M		11	Huang 2005
R5P. infestans	M		11	Huang 2005
R6P. infestans	M		11	El-Kharbotly et al. 1996
R7P. infestans	M	dms	11	El-Kharbotly et al. 1996
R8P. infestans	M	dms	11	Huang 2005
R9P. infestans	M		11	Huang 2005
R10P. infestans	M		11	Huang 2005
R11P. infestans	M		11	Huang 2005
P. infestans	QTL		8	Meyer et al. 1998
P. infestans	QTL		4/5	Leonards-Schippers et al. 1992
P. infestans	QTL	tbr/dms	3/5/6/9/11	Oberhagemann et al. 1999
R12P. infestans	M	tbr/spg	10	Sanchez et al. 2000
R13P. infestans	M	tbr/spg	7	Sanchez et al. 2000
Rpi-ber1 P. infestans	M	brt	10	Ewing et al. 2000
RBP. infestans	M	brt	8	Naess et al. 2000,
		ber		Song et al. 2003
		blb		Van der Vossen et al. 2003
				Van der Vossen et al. 2004
				Park 2005
Rpi-blb1 P. infestans	M	blb	8	Park 2005
Rpi-blb2 P. infestans	M	blb	6	Sandbrink et al. 2000
Rpi-blb3 P. infestans	M	blb	4	Bisognin et al. 2005
Rpi-abpt P. infestans	M		4	Smilde et al. 2005
P. infestans	QTL		4/10	Smilde, unpublished results
P. infestans	QTL	mcd		Smilde, unpublished results
Rpi-mcd1 P. infestans	M	mcd		Smilde, unpublished results
Rpi-neo P. infestans		mcc	9	Ghislain et al. 2001
Rpi-oka1 P. infestans		neo	4/7	Kuhl et al. 2001
Rpi-oka2 P. infestans	M	oka	9	Park 2005
P. infestans	M	oka	4	
Rpi1P. infestans	QTL	phu	7/12	
R2-likeP. infestans	M	pnt	7	
	M		4	

Virusresistance Namevirus		type	source	chromosomereference
Rx1 (Rx)PVX		M	adg	11Hämäläinen et al. 1998
		M	adg CPC 1673*	12Ritter et al. 1991,
				Bendahmane et al. 1997
				Kanyuka et al. 1999
			acl MPI 44.1016/10 5	Ritter et al. 1991
Rx2	PVX	M	tbr5	de Jong et al. 1997
Nb	PVX	M	phu, IVP35	9Tommiska et al. 1998
Nxphu	PVX	M	DE 83-3121	8Marczewski et al. 2002
Ns	PVS	M		Vidal et al. 2002
R Y1	PVY	M	adg	11Hämäläinen et al. 1997
Rysto	PVY	M		Sorri et al 1999
				Kasai et al. 2000
			sto	11Brigneti et al. 1997
			chc	9Hosaka et al. 2001
Rysto	PVY	M	tbr	4F. Celebi-Toprak et al.
Rychc	PVY	M		2002
Nytbr	PVY	M	chc/yun	11Marczewski et al. 2001
			chc/yun	11Marczewski et al. 2004
PLRV.1 PLRV		QTL		
PLRV.4 PLRV		QTL		

^pedigree not certain

The objective of this thesis is the development and implementation of molecular markers linked to the resistance against the root-knot nematodes Meloidogyne hapla, M. fallax and M. chitwoodi. The ultimate goal is the implementation of MAS in potato breeding in order to develop potato cultivars with resistance to these root-knot nematodes. The research was focussed on qualitative monogenic resistance as well as on quantitative resistance. In addition to the genetic analyses of the resistance, differential interaction with nematode populations was performed to predict the durability of the resistance. Chapter 2 describes the mapping of the quantitative resistance to M. hapla and M. fallax in a S. chacoense hybrid. The resistance genes detected as described in Chapter 2 are tested to different M. hapla populations in Chapter 3. Chapter 4 reports the mapping of another quantitative resistance against M. hapla in a S. tarijense hybrid. The mapping of the monogenic resistance against M. chitwoodi and M. fallax in S. fendleri is described in Chapter 5. In that report an interspecific and an intraspecific mapping population are used and results compared. Chapter 6 describes the mapping of another monogenic resistance gene to M. chitwoodi and M. fallax from S. hougasii and the variability of different M. chitwoodi populations to the resistance genes detected in S. bulbocastanum, S. fendleri and S. hougasii. Finally the implementation of markers linked to the detected resistance genes is discussed.

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Chapter 2

Towards marker assisted selection for Solanum chacoense derived resistance against root-knot nematodes (Meloidogyne spp.) in cultivated potato.

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Abstract

The wild potato species Solanum chacoense contains resistance to the root-knot nematode species Meloidogyne fallax and M. hapla. To map the quantitative trait loci (QTLs) for this resistance, a genetic map of a segregating BC1 population was generated based on a total of 623 AFLP markers. Three QTLs were identified in this population. Two QTLs, $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}chcB}$, were explaining 38% and 13% of the total variance of the resistance to M. hapla. One QTL, $R_{Mf\text{-}chc}$, for resistance to M. fallax was identified, explaining 14% of the total variance. The map position of the QTL for resistance to M. fallax did not coincide with the positions of the QTLs for resistance to M. hapla. Due to the large differences in AFLP patterns between S. chacoense and S. tuberosum the identification of the linkage groups to potato chromosome nomenclature could not be established. Consequently chromosome numbers could not be assigned to the linkage groups containing the QTLs.

Introduction

The economic losses in potato cultivation due to nematodes are mainly caused by Globodera spp. and Meloidogyne spp. (Phillips 1994, Oerke et al. 1994). Resistance to nematodes is a major objective in potato breeding as a result of the reduced application of nematicides (Santo & O'Bannon 1981). Currently Globodera infestation is largely controlled by the combination of crop rotation and the cultivation of resistant potato varieties. Unfortunately Meloidogyne resistance is not available in cultivated potato and crop rotation does not offer a solution for Meloidogyne spp., because of their polyfagous nature. In an earlier study a Solanum chacoense accession with a combined resistance against M. hapla and M. fallax, but susceptible to M. chitwoodi was identified (Janssen et al. 1997a). The same paper describes that the resistance to M. fallax in Solanum spp. was generally combined with resistance to M. chitwoodi. The localisation of the resistance gene against M. fallax on the same map position as the M. chitwoodi resistance gene, mapped by Brown et al. (1997) in S. bulbocastanum derived material, suggests that M. chitwoodi and M. fallax share virulence factors (Rouppe van der Voort et al. 1999). The absence of the generally combined resistance against M. chitwoodi and M. fallax in S. chacoense suggests that this resistance is effective to a different virulence spectrum. The introduction of this resistance is therefore desirable in potato breeding. However, introgression breeding requires time and labour consuming phenotypic screening for root-knot nematode resistance. In addition M. fallax is a quarantine organism, which puts certain restrictions to the bioassay. Application of molecular markers for the selection of resistant offspring genotypes offers the advantages of a rapid and non-laborious screening for resistance.

This paper describes the first step towards the development of marker assisted selection for the resistance. First, the inheritance of the S. chacoense derived resistance is studied. Second, the loci involved in resistance are mapped and finally the prospects of marker assisted breeding are discussed.

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Materials & Methods

Plant material

The diploid (2n=2x=24) mapping population RH96-378 was obtained from a cross between the susceptible *S. tuberosum* female parent RH91-183-8 and the resistant male parent 87-206-6, an interspecific hybrid between *S. chacoense* accession BGRC18618 and the diploid *S. tuberosum* clone SH83-81-47. In previous experiments the hybrid 87-206-6 displayed a high level of resistance against *M. hapla* and was very vigorous (Janssen et al. 1996b). Approximately 1000 seeds were sown in vitro. The first germinating and most vigorous seedlings were multiplied in vitro to allow replicated resistance tests, genotype maintenance and tissue generation for DNA extraction. This resulted in a mapping population of 261 individuals.

Inoculum

Out of different race A types of *M. hapla*, the isolate Hi was chosen for the resistance test because of its high virulence (Van der Beek et al. 1998a). The *M. fallax* isolate Fb chosen for the resistance test was originally obtained from a soil sample in the Dutch town Baexem. Due to the lack of genetic variation within *M. fallax* (Van der Beek et al. 1997) the Fb population is assumed to be representative for all *M. fallax* populations in the Netherlands. The nematode populations were propagated on *Lycopersicum esculentum* cv. Motelle and were kindly provided by Dr. Hans van der Beek (Plant Research International, Wageningen).

Meloidogyne resistance test

The in vitro propagated cuttings were transplanted in clay pots of 350 cm^3 containing silver-sand and a slow release NPK fertiliser (Osmocote). During the first six days the relative humidity was kept close to 100%. The plants were grown in a greenhouse with a temperature ranging between 18 and 25° Four plants per genotype were tested for resistance against *M.C. hapla* and another four plants for resistance against *M. fallax* in a randomised block design. For the resistance test with *M. hapla*, there was insufficient plant material of the parents available. The resistant and susceptible phenotype of the parents was confirmed in a later experiment. In the resistance test with *M. fallax* both parents were included in 8 replications. In both resistance tests the susceptible variety Nicola was included to compare the number of egg masses with the amount of egg masses formed in the mapping population.

Four weeks after transplanting the in vitro plants into pots, the plants were inoculated with 1 ml of a water suspension containing approximately 500 second stage juveniles (J2s). Eight weeks later the root systems were rinsed with water to free them from sand and the egg masses were stained with phloxine-B (Dickson & Struble 1963) and counted. The numbers of egg masses was considered to be a measure of susceptibility and resistance. The size of the root systems was scored in 5 classes, ranging from 1: poorly developed to 5: very vigorously. Plants with a class 1 root system were discarded from further analysis.

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DNA isolation and the AFLP protocol

DNA was isolated from frozen leaf material using a modified protocol of Van der Beek et al. (1992). The AFLP protocol was followed as described by Vos et al. (1995), except for the EcoRI adapter, which was used without the biotin label. The restriction enzymes EcoRI and MseI were used for the digestion of the genomic DNA. The EcoRI primers were radioactively labelled and the amplified fragments were visualised by autoradiography. The primer combinations used were E+AAA/M+ACG, E+AAC/M+CAC, E+AAC/M+CAG, E+AAC/M+CCA, E+AAC/M+CCT, E+AAG/M+CCT, E+ACA/M+CTA, E+ACC/M+AAG, E+AGA/M+CAT, and E+ATG/M+CTA. Six out of the ten primer combinations used in this study were also used to make a catalogue of AFLP markers covering the potato genome (Rouppe van der Voort et al. 1998a).

Data collection and linkage map construction

Autoradiograms with AFLP fingerprints were analysed visually with the help of the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). This computer program simplifies the data collection and transforms the collected data in compatible data for Joinmap 2.0 (Stam & van Ooijen 1995), the computer program used to construct the genetic linkage maps. The genetic data were divided based on female and male gamete segregation in order to construct a paternal and maternal linkage map. Co-dominant AFLP markers segregating from both parents were scored dominantly and are therefore less informative as compared to the 1:1 markers. Those co-dominant markers, so-called bridge markers, segregating in a 3:1 ratio, were only added to the data set to align the two maps. For the analysis of marker distances those markers are omitted. After the determination of the marker order, the data were checked for the occurrence of singletons. All AFLP fragments giving rise to a singleton were inspected and if necessary rectified. The linkage groups were assigned to the potato chromosomes using locus specific AFLP markers from an online catalogue of AFLP markers (http//:www.dpw.wur.nl/pv/aflp/cataIog.htm; Rouppe van der Voort et al. 1998a).

Statistical analysis

To normalise the data of the resistance tests, a square root transformation was performed on the number of egg masses counted per root system. Analysis of variance was carried out on the normalised data. The wide sense heritabilities (h^2) of the two resistance tests were calculated, with the formula $h^2 = \int^{2}(y) / [\sigma^2 (g) + \int^{2} (e)]$.

The interval mapping method of MapQTL 4.0 (Van Ooijen & Maliepaard 2000) was applied to determine the LOD score and percentage of explained variance for the quantitative data of the nematode resistance. For the QTL analysis a selection of markers was made in such a way that a proportional distribution of markers over the linkage groups was obtained, with a mean interval length of approximately 5 cM. To establish an appropriate threshold value for declaring a significant QTL effect, the permutation test (Churchill & Doerge 1994) was performed as it is now available in MapQTL 4.0. The permutation test implies that the phenotypic data are shuffled, analysed for QTL effects at all markers in the set and the score saved. This procedure was repeated 1000 times. The 95% quantile was taken as an empirical obtained critical value at which the overall type I error rate for the experiment was 0.05 or less. If the LOD score exceeds this threshold, the presence of a QTL is inferred.

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Results

Resistance tests

The number of egg masses observed in the progeny varied from zero to 107 and from zero to 143 for *M. hapla* and *M. fallax* respectively. In the resistant parent 87-206-6 the average number of egg masses over four replications was 0.5 egg masses of *M. hapla* and 0.0 egg masses of *M. fallax*. The mean number of egg masses formed on the susceptible clone RH91-183-8 was 37 and 64 for *M. hapla* and *M. fallax* respectively, whereas the susceptible potato variety Nicola showed 103 egg masses of *M. hapla* and 122 egg masses of *M. fallax*.
The average number of egg masses over the 4 replications differed significantly ($P<0.001$) between clones. The h^2 of the resistance against *M. hapla* and *M. fallax* was 53 % and 17 % respectively. No correlation between *M. fallax* and *M. hapla* resistance was observed. The frequency distribution of the mean square root of the counted egg masses of the progeny is shown in Figures 1 and 2. For both resistances the trait-values show a continuous distribution.

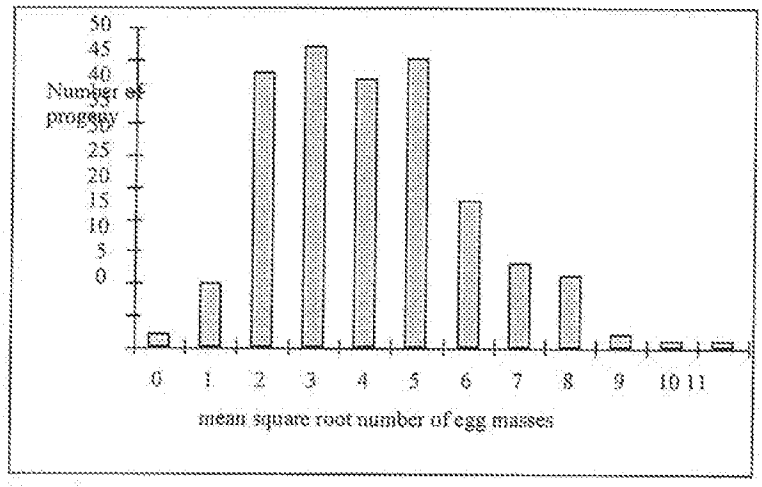


Figure 1: Frequency distribution of the mean square root of egg masses of *M. hapla* detected on the RH96-378 mapping population.

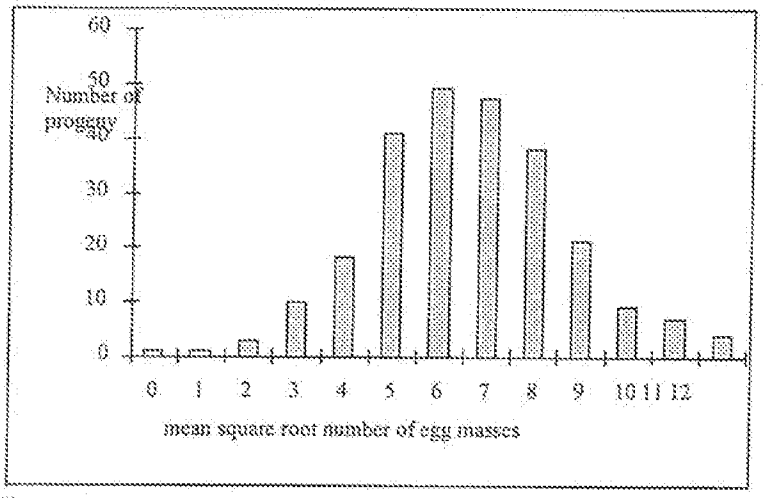


Figure 2: Frequency distribution of the mean square root of egg masses of *M. fallax* detected on the RH96-378 mapping population.

Linkage map construction

The use of 15 primer combinations revealed 313, 284 and 26 markers segregating from respectively the susceptible female parent, the resistant male parent and both parents. At a LOD score of 7 the maternal as well as the paternal markers group into 12 linkage groups with a total length of 685 cM and 596 cM respectively. In the map of the susceptible female parent 7 markers remained unassigned and in the map of the resistant male parent 9 markers remained unassigned. A high level of skewed segregation was observed, as only 44% of the markers fitted the 1:1 ratio ($P < 0.05$). The majority of the distorted segregations is from the hybrid parent (68%), in favour of the S. chacoense alleles. The similarity of the AFLP fingerprints between the resistant interspecific hybrid and the susceptible S. tuberosum clone was low. Approximately 4% of all polymorphic markers segregated from both parents. Usually the proportion of co-dominant markers in diploid potato crosses is ranging from 10% to 30%. Due to the low number of co-dominant markers, only five parental linkage groups could be aligned.

Chromosome alignment

Rouppe van der Voort et al. (1997a) state that when AFLP fragments have an equal electrophoretic mobility and target on the same genomic region, they are "common AFLP markers" and can be regarded as allelic. The alignment of the linkage groups to the potato chromosomes by this method was unsuccessful. Using the reference parents of the catalogue, only 51 of the 60 co-migrating markers of the female parent were common AFLP markers (Table 1). By means of those common AFLP markers 10 linkage groups of the susceptible parent could be assigned to a chromosome number. No linkage groups were assigned to chromosome 3 and 12. None of the 22 co-migrating markers of the interspecific hybrid resulted in an unequivocal identification of the linkage groups and consequently the chromosome numbers could not be assigned to the linkage groups using the catalogue. By means of the co-dominant markers five linkage groups of the interspecific hybrid are aligned with linkage groups of the susceptible parent. Those are assigned to chromosomes 4, 7 and 11, and the two unassigned linkage groups, chromosome 3 and 12.

Table 1: Number of co-migrating AFLP markers between the two parental clones and the 5 reference parents, with seven primer combinations. Some AFLP markers are present in more than one reference parent.

Parental genotypes	CEA	MB	HS	H	Total
RH91-183-8	10	12	15	23	24	60
96-206-6	3	4	4	8	9	22

Localisation of QTLs involved in resistance

Because of the continuous variation of the amount of egg masses of M. hapla and M. fallax, it was impossible to make a clear segregation in resistant and susceptible progeny. Therefore the normalised data were used for quantitative trait mapping. For QTL analysis a selection of the markers was made in such a way that the mean interval length was approximately 5 cM to get a proportional distribution of markers over the linkage groups. The threshold value for declaring a significant QTL, calculated by the permutation test, was set on a LOD score of 4.4 and 4.2 overall for M. hapla and M. fallax. If the LOD score exceeds this threshold, the presence of a

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QTL is inferred. The three linkage groups on which significant QTLs were detected are shown in Figure 3.
One QTL for resistance against M. fallax was identified, explaining 14% of the total variance. The two QTLs for the resistance to M. hapla were explaining 38% and 13% of the total variance. Two factor analyses of variance based on the amount of egg masses of M. hapla gave a significant interaction ($P<0.01$) between the two QTLs. In spite of its significance, the effect of this interaction was very small, so we may assume that the two genes generally act in an additive way. After the first map construction the QTL that explained 38% of the variance was mapped in an interval of 33.2 cM. In order to map the resistance gene more accurately, more AFLP markers were added to fill the gap. The primer combinations were selected via Bulked Segregant Analysis (Michelmore et al. 1991), with a bulk composed of 8 plants that amplified a band for both flanking markers and a bulk with 8 plants lacking the amplified band for both markers. Nine primer combinations out of 36 tested primer combinations, gave a bulk specific polymorphism, of which the 6 best scorable primer combinations were tested on the whole population. The addition of these markers to the linkage group lead to a smaller but still large interval of 17.1 cM. None of the 313 AFLP markers segregating from the susceptible maternal parent showed significant association with resistance.

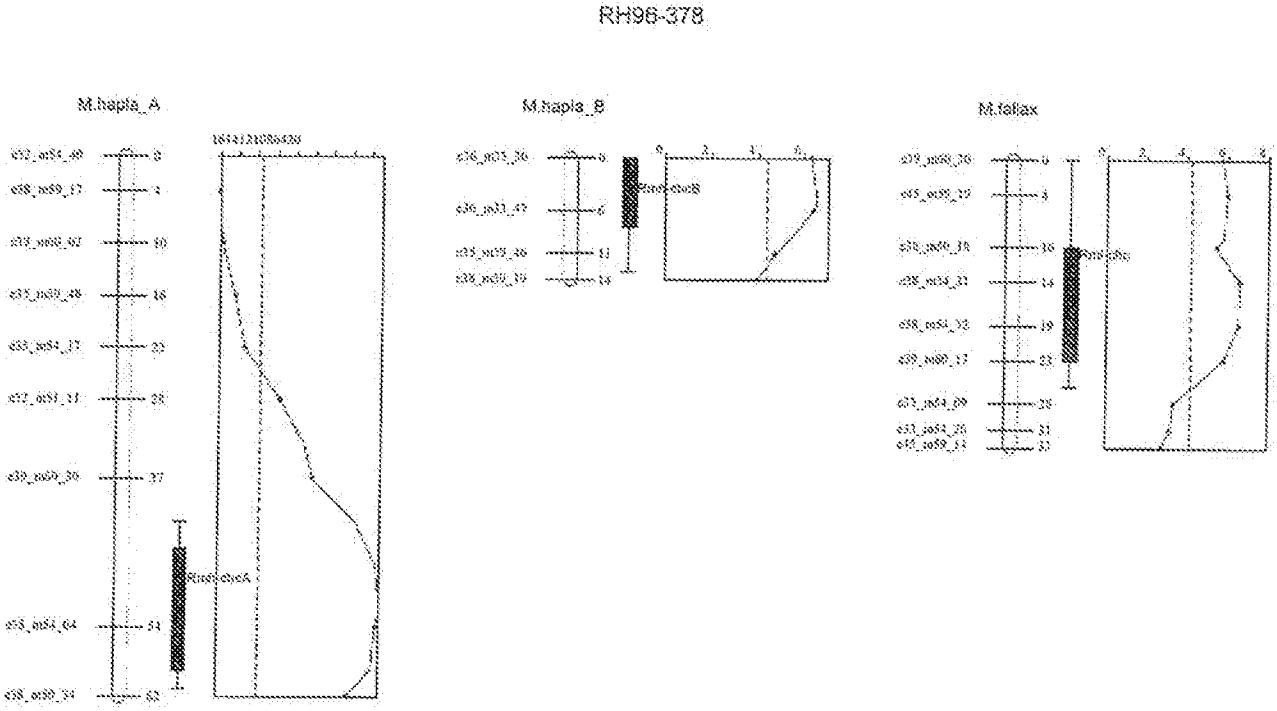


Figure 3: Three linkage groups of the RH96-378 mapping population with the positions of the QTLs $R_{Mh\text{-}chr}A$ and $R_{Mh\text{-}chr}B$, explaining respectively 38% and 13% of the phenotypic variance for resistance to M. hapla, and the QTL $R_{Mf\text{-}chr}$ explaining 14% of the phenotypic variance for resistance to M. fallax. Marker names are based on the primer combination and an arbitrary number. Distances between markers are given in centiMorgans. The QTLs detected by interval mapping are indicated with bars.

To detect minor QTLs the Multiple-QTL model (MQM) mapping method (Jansen 1994) of the software program MapQTL4.0 (Van Ooijen & Maliepaard 2000) was applied using the markers flanking the largest QTLs as cofactor. Only for the resistance to M. hapla a novel significant effect was detected that explained 9.6% of the variance. This QTL mapped on chromosome 7 and coincides with a QTL for root size. The positive correlation between the size of the roots and the number of egg masses was already noticed in previous statistical analyses (results not shown). We assume therefore that this QTL is involved in root-growth and consequently influences the amount of produced egg masses in a genotype.

The two QTLs conferring resistance to M. hapla allow a classification of offspring genotypes into a group having both QTLs, two groups with one QTL and a group without QTLs as shown in Figure 4. The average number of egg masses of the class with both QTLs, with the QTL having the largest effect, the QTL having the smaller effect and without a QTL are respectively 7.1, 11.6, 20.7 and 36.4 eggmasses. Within the classes there was a wide range of egg mass production. Genotypes of the four different classes also showed a big overlap in the mean number of egg masses produced.

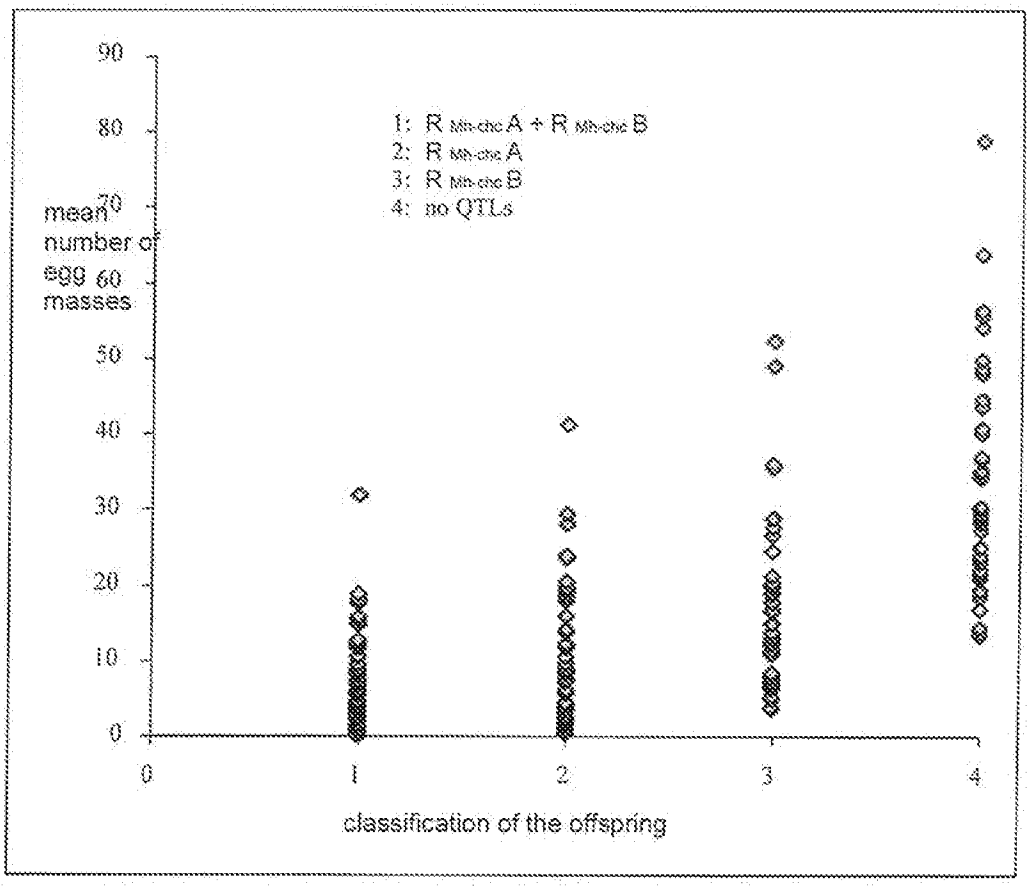


Figure 4: Mean number of egg masses per classification based on the genetic configuration of the offspring.

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Discussion

The resistance to M. hapla of the clone 87-206-6, previously described by Janssen et al. (1996b), is confirmed in this research. The hybrid showed also resistance to M. fallax, comparable with the resistance of other S. chacoense accessions tested in that previous research. The relatively low number of egg masses in the susceptible parent can be explained by the small root system of genotype RH91-183-8. Root-size strongly affects the amount of produced egg masses on the plant and thus hampers the detection of true resistance genes.

In the mapping population a continuous distribution of the numbers of egg masses was found. Such a continuous distribution is expected when the resistance is controlled by QTLs. Another reason for the continuous distribution is the influence of environmental factors. High environmental variation and consequently low heritability of M. fallax resistance could cause to a large extent the continuous distribution of the number of egg masses found. Another factor causing a continuous distribution might be the heterozygosity of the M. hapla population. Van der Beek (1997) found within isolates of M. hapla a comparable large genetic variation as between isolates. When a M. hapla population is very heterozygous, it is plausible that it is also heterozygous for the avirulence genes. A mixture of nematodes with different avirulence genes can not only explain the continuous distribution but can also explain the low heritability. A continuous distribution of egg masses produced makes it impossible to form distinct groups of resistant and susceptible individuals and the results of such a resistance test should consequently be analysed with a QTL approach to localise the genetic factors of the resistance.

In order to perform the QTL analyses a genetic map was made with a total map length of 685 cM and 596 cM for respectively the female and the male map. The lengths of these maps are slightly smaller than other published genetic maps (Jacobs et al. 1995, Rouppe van der Voort et al. 1997b, Gebhardt et al. 1994), but we expect to have covered the potato genome sufficiently to allow the detection of all QTLs involved in these resistances. Seven out of 12 linkage groups of the resistant parent were to a large extent, or even completely, distorted. It was not unexpected that the linkage groups would have regions that show distorted segregation, because a large number of seeds did not germinate and approximately 20% of the seedlings did not form normal leaves after the cotyledon stage and were consequently discarded from further analysis.

The expectation was that approximately half of the polymorphic AFLP fragments of the interspecific hybrid 87-206-6 originated from S. chacoense and the other half from S. tuberosum. However over 80% of the polymorphic AFLP fragments was S. chacoense specific (results not shown). We don't have an explanation for this high amount of S. chacoense specific polymorphic AFLP fragments in the interspecific hybrid, by which as a consequence the AFLP fingerprints between the hybrid and the S. tuberosum clone largely differ. Due to this large difference in AFLP fingerprints "the comigrating AFLP marker approach" could not be used to assign chromosome numbers to the linkage groups. This was also the case in a mapping study with a S. bulbocastanum background (Rouppe van der Voort et al 1999). Clearly it is important that the Solanum species investigated is not too diverged from S. tuberosum to use "the co-migrating AFLP marker approach".

The results of QTL mapping show only one locus for M. fallax resistance, mapped on one of the distorted linkage groups. We propose to name this locus $R_{Mf\text{-}cha}$ in accordance to the proposed nomenclature for R-genes (Chapter 6). The linked markers showed a skewed segregation in favour of the resistant allele. In spite of the detection of the QTL in the majority of the genotypes of the mapping population, hardly any of these genotypes reached the resistance level of the resistant parent. Although the level of resistance is hardly maintained in the offspring, its value might still be useful for potato breeding. The S. chacoense resistance to M. fallax seems to differ in spectrum of resistance found in other Solanum spp.. In most cases resistance to M. fallax coincides with resistance to M. chitwoodi, however S. chacoense is susceptible to M. chitwoodi (Janssen et al. 1996a). The markers flanking the QTL for M. fallax resistance could be used for marker assisted selection (MAS). Taking into account the very low heritability that hampers the phenotypic selection and the assumed different resistance spectrum of S. chacoense, the introgression of $R_{Mf\text{-}cha}$ by means of MAS would be justified.

By means of QTL analyses two major QTLs were detected conferring resistance to M. hapla. The proportion of the phenotypic variation explained by these QTLs is comparable with the heritability, which is the proportion of the genotypic variance relative to the phenotypic variance. This suggests that the genome was sufficiently covered with markers and the offspring size was effective to identify all relevant loci involved in resistance. We name the resistance genes $R_{Mh\text{-}cha}A$ and $R_{Mh\text{-}cha}B$. According to the proposed nomenclature for R-genes (Chapter 6) suffixes could be added to the name, to indicate also the map position. Until a chromosome number is definitive assigned to the linkage groups we propose to add a letter to distinguish both QTLs. The two QTLs allow a classification of the offspring into four different groups having both $R_{Mh\text{-}cha}A$ and $R_{Mh\text{-}cha}B$, only $R_{Mh\text{-}cha}A$ or $R_{Mh\text{-}cha}B$ and a group without the two QTLs as shown in Figure 4. One plant seems phenotypically susceptible but is characterised as having both resistance genes. This genotype might be the result of double crossover event. With other nematode resistance genes it is known that even in shorter intervals such double crossover events seem to occur, although the susceptible phenotype might also be a result of genetic instability of the resistance alleles (Ballvora et al. 1995).

Figure 4 also demonstrates that phenotypic selection will lead to a frequent loss of one of the QTLs. Therefor the use of Marker Assisted Selection should be preferred, in order to decrease this risk. Based on a similarity study of Hospital & Charcosset (1997) the population size needed for introgression of resistances using molecular markers can be estimated. In that study the minimum population size required to obtain at least one individual with the resistance gene and all markers is calculated. A reasonable high value of this probability can be obtained in the first generation with no more than two markers per QTL. Implementing the results of the present study in the formulas presented by Hospital & Charcosset (1997) lead to high probabilities of having the desired genotype at the QTLs with a minimum population size of 21. A fast introduction of these QTLs by means of MAS should therefore be feasible, resulting in partial resistant commercial varieties.

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Chapter 3

Differential interaction of the root-knot nematode Meloidogyne hapla with Solanum chacoense derived resistance genes

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Abstract

The differential interaction of eight M. hapla populations to the resistance QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ from the wild potato species S. chacoense and the molecular variation of the M. hapla populations were studied. The $R_{Mh\text{-}chc}A$ allele allows recognition on virulence factors present in the six tested cytological race A populations. Only in the population initially used for the QTL analysis, an additive effect of the minor QTL $R_{Mh\text{-}chc}B$ was found. No effect of both alleles was noticed for the two race B populations. Knowledge of these virulence factors in the nematode populations and the complementary resistance genes are tools for identification and resistance breeding. Based on the molecular variation of the M. hapla populations the cytological races A were divided in a different group than the populations of race B. The cytological races A and B can thus be divided based on their differences in virulence to the $R_{Mh\text{-}chc}A$ allele and based on molecular differences. The use of a pathotype scheme and the gene pool similarity concept are discussed.

Introduction

Root-knot nematodes Meloidogyne spp. are globally distributed pathogens, of which M. hapla is most commonly found in areas of potato cultivation and can cause severe yield reduction (Brodie 1984, MacGuidwin & Rousse 1990). These losses can be restricted by crop rotation; however the wide host range of M. hapla limits a practical application of this strategy. The use of nematicides seems a more consistent way of controlling the root-knot nematode, but is environmentally unfriendly and should be avoided as much as possible. The most desirable and effective method of nematode control is the use of resistance. Unfortunately no acceptable level of resistance is found in commercial potato cultivars. Recently, in the wild potato species Solanum chacoense two QTLs were detected for resistance to the M. hapla population Hi (Chapter 2).

The durability of this resistance is depending on the genetic diversity for genes involved in virulence of the nematode and the selection for virulent genotypes upon exposure of specific resistance genes. In potato isolate-by-cultivar interaction was detected, suggesting the existence of variation in virulence genes in M. hapla (Van der Beek et al. 1998a). Also several factors involved in (a)virulence were detected in a study with wild Solanum species (Van der Beek et al. 1998b). The two races of M. hapla: race A which is facultative meiotic parthenogenetic and race B that is obligate mitotic parthenogenetic, show both a similar range of (a)virulence genes (Van der Beek et al. 1998b). Assuming that a number of different (a)virulence genes exist in M. hapla, it is important to arrive at a reliable pathotype scheme that can help potato breeders in pyramiding resistance genes in cultivars to cover a broad spectrum of virulence factors. For that purpose the nematode populations should be assigned to pathotypes based on a combination of molecular data and virulence characteristics. Similar studies to reveal the relationship between genetic diversity across nematode populations and

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virulence factors have been conducted in potato cyst nematodes (Blok & Phillips 1995; Folkertsma et al. 1996b; Thiery et al. 1997). In a similarity study based on the two-dimensional gel electrophoresis method little intraspecific variation was observed within different M. hapla populations but a clear separation could be made between race A and race B (Van der Beek et al. 1997).

In this paper we describe experiments to analyse the interaction between M. hapla and two resistance QTLs from the wild potato species S. chacoense as revealed in a previous study (Chapter 2). The variation in virulence will be compared with the molecular variation present in diverse M. hapla populations. An estimation of the genetic diversity of the investigated M. hapla populations will be made based on an AFLP fingerprint.

Materials & Methods

Potato genotypes

Two QTLs, $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}chcB}$, conferring resistance to the M. hapla population Hi have been identified in the mapping population RH96-378 (Chapter 2). RH96-378 is a cross between the susceptible diploid S. tuberosum genotype RH91-183-8 (2n=2x=24) and the resistant parent 87-206-6, an interspecific hybrid between S. chacoense accession BGRC18618 and the diploid S. tuberosum clone SH83-81-47.

Based on the AFLP-data, the individuals of the BC1 population could be divided in four different groups: a group having both QTL-alleles: $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}chcB}$, a group with the $R_{Mh\text{-}chcA}$ allele, a group with the $R_{Mh\text{-}chcB}$ allele and a group without the two alleles associated with resistance at both loci. Initially six genotypes from each group were selected and multiplied in vitro. One genotype from the group having both alleles hardly grew in vitro and was discarded from further examination. One genotype with allele $R_{Mh\text{-}chcB}$ was incorrectly assigned as having no resistance. Another genotype from the group with both QTLs was tested again with AFLP and turned out to lack $R_{Mh\text{-}chcA}$. In the end 4, 6, 8 and 5 genotypes of the group containing both alleles, the $R_{Mh\text{-}chcA}$ allele, the $R_{Mh\text{-}chcB}$ allele and group without the two alleles respectively, were transplanted in clay pots of 350 cm^3 containing silver-sand and a slow release NPK fertiliser (Osmocote). During the first six days in a greenhouse, the relative humidity was kept near 100%. Five plants per genotype were tested for resistance against different M. hapla populations in a randomised block design. The two parents of the genotypes and the susceptible potato cultivar Nicola were included. The transplantation of the in vitro plants into the silver-sand resulted in the death of several plants. Due to the unsuccessful transplantation together with the plants with a poor root development 15% of the plants were discarded from analysis. The remaining plants were analysed statistically.

Nematodes

Eight M. hapla populations have been used in this study (Table 1). They were collected from different hosts and different geographical sites in the Netherlands and from several countries around the world. The 8 populations Ham, Han, Hao, Haw, Hb, Hbs, Hbz and Hi, used in the virulence test were maintained and propagated on Lycopersicum esculentum cv. Moneymaker.

Table 1: The origin and races of the M. hapla populations. The cytological race A and B were distinguished by studying the meiosis (Van der Beek 1997).

Population	origin	Last host	race
Ham	Australia	unknown	A
Han	South Korea	Hosta	B
Hao	Heide, the Netherlands	unknown	A
Haw	France	Grape	A
Hbz	Zwaansbeek, the Netherlands	Astilbe	A
Hbs	Horst, the Netherlands	Fennel	A
Hbz	Amerzoden, the Netherlands	Rose	B
Hi	Smilde, the Netherlands	Tomato cv. Motelle	A

Virulence test

Four weeks after transplanting the in vitro plants, an aliquot of approximately 500 second-stage juveniles (J2s) were inoculated into small holes around the stem of the plants. Eight weeks later the roots of the plants were rinsed free from sand and stained with Phloxine-B (Dickson & Struble, 1965). Individuals with a very small root system were omitted for further examination. The numbers of egg masses were counted as a measure of virulence/resistance.

DNA isolation & AFLP analysis

DNA isolation of the nematodes in J2 stage was performed according to Zijlstra et al. (1995) and checked on agarose gel to determine the DNA concentration. Samples with no visible DNA were precipitated with an equal amount of isoamylalcohol and pelleted in a microcentrifuge. The pellet was dissolved in 10 $\mu\lambda$ deionised water and directly used for AFLP template preparation. From the other samples approximately 50 ng genomic DNA was used for the AFLP template preparation. The AFLP procedure was performed following the protocol as described by Vos et al. (1995). The restriction enzymes used were EcoRI and MseI. The pre-amplification was performed using the primers E+0/M+C, followed by PCR amplification using the M+CAC primer and a ^{33}P-labeled E+A primer. A water control was included in the AFLP analysis. The AFLP autoradiogram was evaluated using the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). The AFLP markers were dominantly scored for each nematode population as 1 (band present) or 0 (band absent) for similarity analysis. A neighbour joining tree was calculated using the computer program TREECON (Van de Peer & De Wachter 1994).

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Results

The virulence of the nematodes and the resistance of the potato genotypes was analysed by counting the egg masses on the root system. The average numbers of egg masses are listed in Table 2. The results of individual plants tested in previous research with the Hi population are included for the sake of comparison, because Hi was the population used to select the QTL in the first place (Chapter 2). The susceptible potato cultivar Nicola was used as susceptible control in this experiment, although there is no guarantee that this genotype is void of R-genes against all populations of M. hapla. Similarly the susceptible diploid clone RH91-183, previously used as a parent to develop the population for the detection of the QTLs involved in resistance against M. hapla, was added. Nicola and RH91-183 proved to be the most susceptible genotypes for the nematode populations Han, Haw, Hb and Hbs. In the case of population Hao and Haw the multiplication on susceptible controls does not exceed the susceptible experimental materials, but the number of egg masses is still in the same range.

A significant effect of the $R_{Mh\text{-}chc}A$ allele could be found in the populations Hi, Ham, Hao, Haw, Hb and Hbs. The $R_{Mh\text{-}chc}B$ allele has a significant effect on the populations Ham and Hi, but no significant effect could be found in population Ham between the three different resistant classes. Only in population Hi the additive effect of the $R_{Mh\text{-}chc}B$ allele was found. No significant effects of the two alleles on the production of egg masses of the two race B populations Han and Hbz could be found. Although the experimental design does not allow statistical analyses of differences in aggressiveness, large variation is detected in the ability to form egg masses on the tested potato genotypes.

Table 2: The average of egg masses of eight different M. hapla populations of race A or B on the genotypes with both resistance alleles, with one resistance allele and without the resistance alleles, and on one resistant (R) and two susceptible (S) controls. For each M. hapla population separately, means carrying a different letter, are significantly different from each other ($P<0.05$).

	race A	race A	race A	race A	race A	race A	race B	race B
	Hi	Hb	Hbs	Haw	Hao	Ham	Han	Hbz
$R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$	1.7 a	2.3a	2.7 a	3.2 a	5.0 a	0.2 a	28.0 a	5.5 a
$R_{Mh\text{-}chc}A$	4.8 b	2.1 a	0.9 a	8.0 a	6.4 a	0.7 a	33.5 a	1.5 a
$R_{Mh\text{-}chc}B$	14.9 c	31.1 b	13.4 b	31.0 b	24.0 b	1.0 a	32.7 a	2.4 a
No resistance	44.3 d	28.28 b	9.7 b	20.6 b	15.0 b	5.1 b	34.7 a	3.6 a
87-206-6 (R)	0.3	0.0	0.2	0.0	0.8	0.0	18.4	0.6
RH91-183 (S)	37.3	77.4	6.0	37.4	10.7	4.3	50.6	0.0
Nicola (S)	103.3	143.6	148.8	23.5	20.8	0.6	37.4	7.0

Molecular diversity across the nematode populations was analysed with molecular markers. The followed DNA extraction method was very laborious and not always succesful.

Unfortunately the DNA of the nematode population Hi could not be isolated. Of the remaining 7 nematode populations one AFLP fingerprint was generated with one primer combination. Because the bands in the upper and in the lower part of the AFLP autoradiogram were not clear in all 7 lanes, only fragments between 100 and 330 basepairs in the middle section of the AFLP fingerprint were analysed. In this section the analysis of the AFLP data revealed 127 clear AFLP fragments of which 99 were polymorphic across the investigated populations. A dendrogram was constructed following the neighbour joining tree method Figure 1. The two nematode populations Han and Hbz are remarkably different as compared to the other populations. The M. hapla race A populations are clustered together.

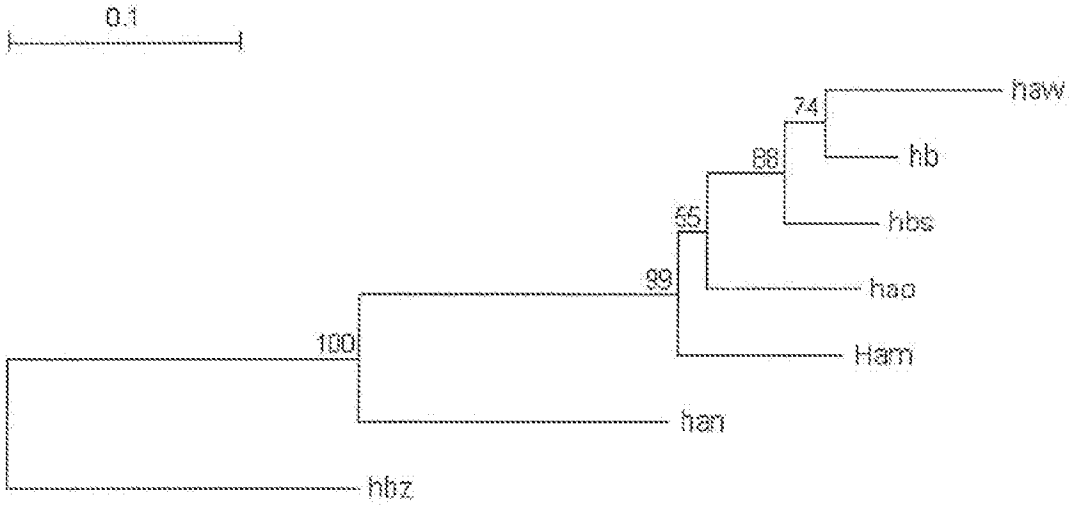


Figure 1: Neighbour joining tree of the seven M. hapla populations based on DNA fragments from AFLP analysis. Percentages of the bootstrap values are mentioned above the branches.

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Discussion

It seems that the $R_{Mh\text{-}chcA}$ allele allows recognition on virulence factors present in the race A populations. No effect of both alleles was noticed for the race B populations. Also the resistant parent was susceptible for the race B population Han, which makes it unnecessary to screen the S. chacoense population with such a race B population. The two races used to be distinguished on their mode of reproduction. With the $R_{Mh\text{-}chcA}$ allele we can now also discriminate between the two races based on their reproduction. The correlation between sexual reproduction and virulence is intriguing, but so far without explanation.

The $R_{Mh\text{-}chcB}$ allele of the minor QTL has an insignificant effect on the M. hapla populations tested. Only in population Hi and Ham there is an effect of the $R_{Mh\text{-}chcB}$ allele causing a reduction of the egg masses produced. It is obvious that this allele should have an effect on population Hi because this was the initial population used to identify and map the QTLs. There is no indication that additional QTLs were present in the S. chacoense mapping population. Although the $R_{Mh\text{-}chcB}$ allele has an effect on population Ham, this allele has no significant additional effect in combination with the $R_{Mh\text{-}chcA}$ allele.

The differences in egg mass production, irrespective of the genetic composition of the individual plant, indicate the variation in aggressiveness between the populations. Indeed large differences in aggressiveness exist between the tested nematode populations. This has also been reported by Van der Beek et al. (1998a) where a race B nematode population showed a much smaller reproduction factor as compared with the other M. hapla populations tested. From a breeders point of view those populations are of little concern. It is unlikely that the growing of resistant varieties would change the population in such a way that it will become more aggressive.

Knowledge of the virulence factors in nematode populations and the complementary resistance genes would provide tools for identification and pyramiding the genes in a variety. Therefore a reliable pathotype scheme would be valuable to the breeder. Kort et al. (1977) proposed a pathotype scheme for Globodera spp. based on the quotient between the final and initial population density (*Pf/Pi* values) on a set of differentials, discriminating five pathotypes of G. rostochiensis and three of G. pallida. This pathotype scheme has been criticized because of the arbitrary use of the *Pf/Pi* values which is environmentally sensitive and because of the use of differential clones with quantitatively based polygenic resistance (Trudgill 1985). G. pallida populations with a similar pathotype, based on the scheme of Kort et al. (1977), are often molecular very distinct (Folkertsma et al. 1994) and they frequently have a different response to various sources of resistance.

An alternative to the pathotypes scheme is the gene pool similarity concept (Bakker et al. 1993) using molecular techniques to establish the similarity between nematode populations. In absence of selection pressure the molecular similarity of the populations will be a reflection of the virulence loci. This concept was confirmed testing the genetic similarities of 102 Dutch

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potato cyst nematode populations and their virulence towards four resistant potato cultivars (Folkertsma 1997). The potato cyst nematodes differ however in dispersal and reproduction with the root-knot nematodes. Considering the broad host range of the root-knot nematode, these nematodes will multiply almost every year and will have more generations each year, whereas the potato cyst nematode will reproduce only during the growth of potatoes and only one generation a year. The dispersal of root-knot nematodes is also higher compared with potato cyst nematodes because they can be dispersed by the tubers, whereas the potato cyst nematode only disperses with the tuber in the attached soil. Due to the higher reproduction and greater dispersal the effects of mutation and selection on a population will be higher compared with the potato cyst nematode. However we expect that the existing virulence characteristics in these populations, before the introduction of resistant cultivars, are the major force behind the "resistance-breaking" nematode populations. Analyses of the virulence related polymorphisms between the nematode populations will therefore predict if new resistance genes have added value.

Although we have limited genetic dataset, large differences between the M. hapla populations have been found. The largest differences have been observed between the two cytological races A and B. We could confirm the results of Van der Beek et al. (1997) and divide the nematode populations of race A in a different group than the race B populations. The cytological races A and B can thus be divided based on their differences in virulence to the $R_{Mh\text{-}Sca}$ allele and based on molecular differences.

One of the aims of this research was to establish the relationships between the diverse populations based on AFLP data in order to use that information in testing the effectiveness of new sources of resistance. Our results show that the breeder has to use race B populations in order to detect new sources of resistance. According to our results the population Han should be preferred because of its combination of virulence and aggressiveness.

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Chapter 4

Genetic analysis of the Solanum tarijense derived resistance to the Northern Root-Knot Nematode Meloidogyne hapla in a potato BC1 population.

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Abstract

A non absolute level of resistance to the Northern root-knot nematode Meloidogyne hapla was observed in the wild Solanum species S. tarijense. In order to perform marker assisted selection for the introduction of this resistance into the susceptible cultivated potato (S. tuberosum), a diploid BC1 mapping population was developed and analysed for its resistance to M. hapla. The mapping population showed a continuous distribution of the number of egg masses. A genetic map was constructed using AFLP markers and the resistance was mapped in a quantitative as well as in a qualitative method. The QTL mapping approach resulted in one locus and this was confirmed later with the qualitative mapping approach. The genetic map was successfully aligned to the genetic map of a catalogue population. The linkage group with the mapped resistance gene could therefore be placed on the distal position of the short arm of chromosome 7. The resistance gene is named $R_{Mh\text{-}tar}$ and is the first described resistance gene mapped on the short arm of chromosome 7.

Introduction

The Northern root-knot nematode Meloidogyne hapla is a common pest in potato producing areas with a temperate climate. It is able to reproduce on a broad range of dicotyledous hosts and can lead to severe economic losses. Potato is a very good host of M. hapla and the nematode can multiply extensively during the growth of potato. Under natural cropping circumstances M. hapla is able to reproduce up to three generations in one growing season of potato. The major damage in potato caused by the nematode is the reduction of yield which can increase up to 70% reduction in a highly infested plot (MacGuidwin & Rouse 1990, Stirling & Wachtel 1985). Furthermore the high number of nematodes that remain in the soil after growing a potato crop will cause severe problems when the next year susceptible crops like carrots or chicory are grown.

In order to control the nematode population the use of nematicides is very effective but for environmental reasons less desirable. Alternative control strategies are crop rotation with monocotyledons and the use of resistant varieties. Minor differences in susceptibility to M. hapla have been observed in commonly grown potato varieties (Van der Beek et al. 1998a), but these differences are too small to be able to select useful levels of resistance. In several wild Solanum species a non absolute level of resistance to M. hapla has been observed, among others in S. tarijense (Janssen et al. 1996a).

Currently, breeding for resistance to M. hapla in potato is relying exclusively on bioassays. A disadvantage of the bioassay is the use of natural nematode populations that are probably a mixture of undetermined races of the nematode. Moreover the bioassay in the greenhouse is expensive and laborious. A far better alternative for the bioassay is the application of molecular marker assays to discriminate between resistant and susceptible plants.

In this paper we describe the genetic analysis of resistance in a diploid population derived from a backcross between S. tuberosum and a F1 hybrid of the wild species S. tarijense. The non absolute level of resistance combined with a continuous distribution of produced egg masses in

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the segregating offspring (unpublished results), suggests that the resistance is under polygenic control. Therefore QTL analysis is pursued using a linkage map well covered with AFLP markers, based on a high number of offspring genotypes. The detection of QTLs is the first step in a marker assisted selection approach, to be used for the introgression of M. hapla resistance in potato.

Materials & Methods

Plant material

The diploid mapping population RHAM061 was obtained from a cross between the resistant parent RH90-011-4 and the susceptible S. tuberosum genotype RH89-039-16. The resistant interspecific hybrid RH90-011-4 is obtained from a cross between S. tarijense (BGRC 24717) and a dihaploid clone of the cultivar Mondial. Seeds of the mapping population were sown in vitro and the seedlings were multiplied in vitro in order to obtain sufficient plantlets for the resistance test and genotype maintenance as well as to generate tissue for DNA extraction.

Resistance test

Three weeks old in vitro cuttings were transplanted in clay pots of 350 cm³ containing silver-sand and a slow release NPK fertiliser (Osmocote), and grown with a relative humidity near 100% during the first six days. The temperature in the greenhouse was maintained at 20°C, with fluctuations of 4° Together with the two parents and 228 offspring, three susceptibleC. potato varieties were included in the resistance test: Bintje, Agata and Donald. Most genotypes were tested in four replications in a completely randomised design. Plants showing inferior growth were omitted from further analysis. Four weeks after transplanting, the plants were inoculated with 1 ml of a water suspension containing approximately 400 J2 nematodes of M. hapla population Hb. Population Hb is a race A of M. hapla that was originally collected from a soil sample near the Dutch town Zwaanshoek (Van der Beek et al. 1998a). The nematode population was propagated on Lycopersicum esculentum cv. Motelle and kindly provided by Dr. Hans van der Beek (Plant Research International, Wageningen). Eight weeks after inoculation the root systems were rinsed and stained with Phloxine-B (Dickson & Struble 1965). The amount of egg masses was determined as a measure of susceptibility. To investigate the data with an analysis of variance, the number of egg masses counted per root system was first normalised with a square root transformation. From the ANOVA the wide sense heritability (h^2) of the resistance test was estimated.

Map construction

The AFLP protocol was followed as described by Vos et al. (1995), using the restriction enzymes PstI and MseI to digest the genomic DNA. The PstI primers were radioactively labelled and the amplified fragments were visualised by autoradiography. All primer combinations were analysed before on the SH×RH population (Rouppe van der Voort et al.

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1997a, 1998a, 1998b; Isodore et al. 2003; http//:www.dpw.wur.nl/pv/aflp/catalog.htm), with RH being the abbreviated name of RH89-039-16. Therefore most of the chromosomal positions of the AFLP markers are already known which enables the assignment of a chromosome number to the different linkage groups. The primer combinations used were P+AC/M+ATC, P+AG/M+AAG, P+AG/M+AAT, P+AG/M+ACC, P+AG/M+AGA, P+AG/M+AGC, P+AG/M+AGG, P+AG/M+AGT, P+CA/M+AGT and P+TG/M+ACT. The analyses of the autoradiograms were done visually with the help of the image analysis software Cross Checker (http//:www.dpw.wur.nl/pv/pub/CrossCheck/). The mobilities of the AFLP markers were estimated, using the mobility of markers mapped in the SH×RH population. The two parental genetic maps were made using the computer program Joinmap 2.0 (Stam & van Ooijen 1995). Therefore the genetic data were divided based on female and male gamete segregation. In order to align the paternal and maternal linkage map co-dominant AFLP markers segregating from both parents were used as bridge markers, segregating in a 3:1 ratio. For the final calculation of map distances the bridge markers are omitted because of their less informative nature.

QTL mapping

The data of the resistance test were analysed with the interval mapping method of MapQTL 4.0 (Van Ooijen & Maliepaard 2000) for QTL detection. The interval mapping method of MapQTL 4.0 determines the LOD scores and percentages of explained variance for the quantitative data. To obtain an even distribution of markers over the linkage groups, a linkage map was constructed with a mean interval length of approximately 5 cM. Using the permutation test (Churchill & Doerge 1994), with a 95% quantile as an empirical obtained critical value at which the overall type I error rate for the experiment was 0.05 or less, a critical LOD value was set. When a LOD profile exceeded this threshold, the presence of a QTL is inferred. In addition to the interval mapping method, the multiple QTL mapping (MQM) module of MapQTL 4.0 was applied. The marker nearest to the largest QTL was taken as a cofactor to eliminate the major part of the variation induced by that QTL and subsequently enhance the detection of minor QTLs on other linkage groups.

In an attempt to locate the resistance locus more precisely, the phenotypic data were analysed qualitatively. A subset of the complete data set was used for this analysis. Only the offspring genotypes of which the data of 3 or 4 replications are known and having a fair root system were used to minimise the chance of false identification of resistant genotypes. The large group of individuals that had a mean number of egg masses between 1 and 10 were also discarded for the qualitative analysis. The trait value below 1 egg mass were considered resistant and values higher than 10 egg masses as susceptible.

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Results

Phenotypic data

The mean number of egg masses produced on the resistant and susceptible parent was 0.5 and 32 respectively. The mapping population showed a continuous distribution of the number of egg masses. The mean number of egg masses counted on the offspring genotypes varied between 0 and 69.25. The most susceptible F1-plant exceeded the number of egg masses formed on the susceptible controls Agata, Bintje and Donald, having a mean of respectively 52, 40 and 33. Analysis of variance of the square root normalised data showed a significant ($P<0.01$) genetic variance in the mapping population. The heritability of the resistance was estimated to be 0.40.

Genetic linkage mapping

A genetic linkage map was constructed using Joinmap 2.0 (Stam & van Ooijen 1995) on the basis of 265 segregating markers on the population of 182 individuals. When the LOD score exceeded 3.0, linkage between AFLP markers was inferred. With this LOD threshold 82 markers (aa×ab) were placed on the RH89-039-16 map, 134 markers (ab×aa) were placed on the RH90-011-4 map and 9 markers remained unassigned. A skewed segregation ratio (χ^2 test, $P<0.001$) was observed in 14%, 20% and 25% of the respectively ab×aa, aa×ab and ab×ab markers. The majority of the markers that show a skewed segregation ratio are linked and mapped on chromosome 6 and 8 of the resistant parent and on chromosome 3, 4 and 12 of the susceptible parent. On both parental maps the markers were evenly distributed, showing hardly any clustering. Because many AFLP markers on the RH89-039-16 map have known map positions, the assignment of a chromosome number to the linkage groups was simple. Subsequently, the identity and orientation of the RH90-011-4 linkage groups could be determined by aligning the two parental maps with 39 out of the 40 ab×ab markers by using these markers as allelic bridges. After the alignment, these markers were discarded for further analysis.

QTL analysis

The interval mapping method showed significant association between markers and resistance on one of the twelve chromosomes of the resistant parent only. The region with this significant effect was located on chromosome 7, explaining 19.7 % of the variation. To determine whether the genetic effects were solely controlled by this region, MQM was performed. The marker P13M41_05 closely linked to the QTL on chromosome 7 was used as a cofactor. No other locus was detected that was significantly involved in resistance using the MQM method.

Qualitative mapping

Besides by means of QTL analysis, resistance was also mapped in a qualitative way. To avoid misclassifications, only the extreme trait values of the resistance test were converted into

qualitative data. The amount of less than1 egg mass for resistant and more than 10 egg masses for susceptibility are used as arbitrary thresholds. The subset of the population contained 88 individuals which is large enough to use for mapping analysis. The resistance is segregating in a Mendelian way 1:1 (χ^2 = 1.64). The resistance analysed with the qualitative mapping approach revealed a linkage group with 12 AFLP markers (Figure 1). In Figure 1 the three bridge markers used to align the two parental linkage groups are included. Three markers, indicated in italics, of the susceptible parent are present in the Ultra High Density (UHD) map of SH×RH (http://www.dpw.wur.nl/uhd/). These markers are placed in so-called Bins. The AFLP marker P+AG/M+AGT_83.8 is mapped in the centromere (Bin 68) of the UHD map. Based on the marker information of the UHD map, the resistance locus can be placed on the distal position of the short arm of chromosome 7. The markers P+AG/M+AAG_32 and P+AG/M+AGG_05 are closely linked to the resistance. Recombinants between the two markers and the resistance gene are present, however no feasible solution in marker order could be found. Therefore the resistance gene and both markers are presented as one locus. Further research is needed to determine the correct ordering of these loci.

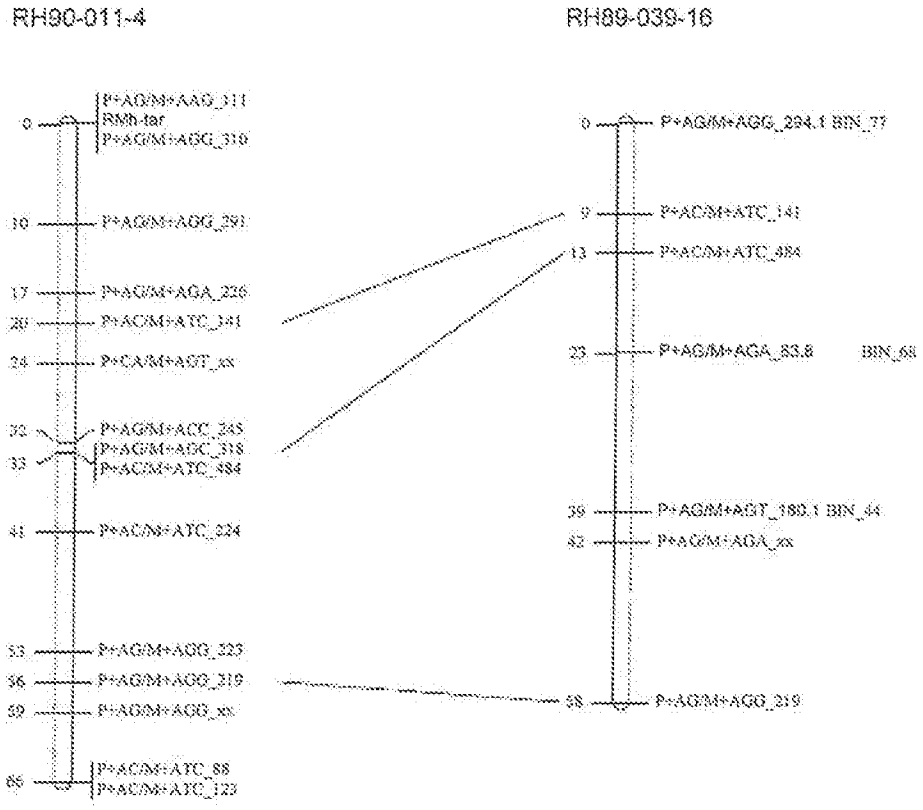


Figure 1: The genetic map of chromosome 7 of the resistant parent RH90-011-4, including the resistance locus R_{Mh_tar}, and the susceptible parent RH89-039-16. The AFLP markers mapped in the SH×RH population are indicated in italics. The Bin numbers that contain these markers are mentioned behind the marker names. Chromosome orientation is according to Dong et al. (2000). AFLP-markers of which the sizes are not estimated are marked by xx.

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Discussion

The estimated broad sense heritability of 0.40 indicates that the amount of egg masses is highly variable and probably strongly influenced by the environment. Based on the low heritability one might conclude that the resistance test was not reproducible. Such low heritabilities have been found in previous resistance tests used to map M. hapla resistance in another population (Chapter 2). There are many environmental factors influencing the outcome of a resistance test, although the complete randomisation of the test should minimise these effects. Clearly these low heritabilities show that the use of a bioassay for estimating resistance to M. hapla is not suitable in a potato breeding program. Therefore the results underline the necessity of linked molecular markers in order to apply marker assisted selection in the breeding program.

The high variability of the resistance test is also a drawback in the mapping of the resistance. Moreover the results show that on the resistant parent some egg masses are formed as was already noticed by Janssen et al (1996a). In their research on average 1.4 egg masses were observed on the resistant parent, where 0.5 egg masses were observed in this study. The successful propagation of a limited number of nematodes on the resistant parent can be explained when assuming the presence of a low frequency of virulent nematodes in the initial population. The nematode population used in this experiment was obtained from a soil sample and could be a mixture of nematode genotypes with different (a)virulence genes. However the current lack of resistance in potato makes it difficult to specify this variation in virulence.

In order to localise the gene(s) involved in resistance a genetic map was constructed. This map was used both for MapQTL analysis as for Joinmap analysis. The diploid potato clone RH89-039-16 was used as susceptible parent because of its existing AFLP map, which is an advantage in map construction. The AFLP markers could be divided easily among the 12 linkage groups based on the previous mapping studies of RH89-039-16 (Isodore et al. 2003; http://www.dpw.wur.nl/pv/aflp/catalog.htm). The locus specific AFLP markers made it easy to assign the correct chromosome numbers to the linkage groups. The resistant parent map could be aligned straightforward by using the ab×ab markers as allelic bridges. This mapping study resulted in two genetic maps of 786 cM and 606 cM for respectively the resistant and the susceptible parent. These map lengths are, although slightly shorter, comparable with the map lengths presented in other studies (Rouppe van der Voort et al. 1997a, 1998a; Celis-Gamboa et al. 2002). The results show distorted markers in both parents, which is a common feature in potato (Jacobs et al. 1995, Bonierbale et al. 1994). By choosing the PstI restriction enzyme as a rare cutter for template preparation, we observed hardly any centromeric clustering of AFLP markers as compared with EcoRI based markers (Van Eck et al. 1995, Rouppe van der Voort et al. 1997a). This decreased clustering of markers was also noticed in other crops like maize (Vuylsteke et al. 1999).

With respect to the covering of AFLP markers over the twelve chromosomes, we assume to have a suitable genetic map to perform MapQTL. The genetic map combined with the phenotypic data should allow the detection of all loci involved in resistance. However the results of the QTL analysis did not indicate that the resistance to M. hapla was controlled in a polygenic way. It was surprising that only one interval with significant association with the resistance was detected. The finding of this QTL did not explain all the genetic variation present in the mapping

population. However also with the multiple QTL mapping (MQM) method (Jansen 1994), which greatly enhances the power of detection of minor QTLs, no other QTLs contributing to the resistance could be detected. This does not imply that other QTLs are absent in this mapping population. Minor QTLs may stay unnoticed, especially in view of the large amount of residual variance caused by the environmental influence on the resistance. Most likely the value of other QTLs, if present in this population, will be of minor importance for breeding purposes considering the impossibility of mapping them.

The detection of just one QTL explaining the resistance, justifies the attempt of mapping this resistance gene by treating the resistance as a qualitative trait. The arbitrary threshold of 1 egg mass represents 0.25% successful reproduction of the total number of inoculated juveniles, which is comparable with other research. Janssen et al. (1996a) used a threshold of 1% reproduction of the total number of inoculated juveniles to distinguish between resistance and susceptibility. Using the subset of 88 individuals the resistance gene could be mapped more precisely. In this research the marker data of the UHD map could be successfully used in order to determine the chromosome number and the orientation of the linkage group. The resistance locus is on the distal position of the short arm of chromosome 7. The orientation of the linkage group as presented in Figure 1 is inverted relative to most of the literature references where TG20 is north and TG61 is south. However, the north and south arm of chromosome 7 as presented in Figure 1 correspond cytogenetically with the long and short arm respectively, according to Dong et al. (2000). Previously only a few other resistance genes have been mapped to this linkage group: the potato cyst nematode resistance Grof (Barone et al. 1990) and S. berthaultii (Sanchez et al. 2000), S. pinnatisectum (Kuhl et al. 2001) and S. phureja (Ghislain et al. 2001) derived late blight resistance. According to the proposed nomenclature for R genes (Chapter 6), we will name the resistance gene $R_{Mh\text{-}1at}$. In order to use a marker assisted selection (MAS) approach for the introgression of this resistance gene in potato more closely linked markers are needed.

Considering the low heritability of the resistance test, a resistance gene like this should be incorporated in potato with MAS instead of the phenotypic bioassay. The practical value of the $R_{Mh\text{-}1at}$ resistance gene for potato cultivation will depend on the allele frequency of a matching virulence within the M. hapla populations and the distribution of such nematode populations. In order to develop resistant potato varieties with a broad resistance spectrum against different M. hapla populations, it is recommended to combine the $R_{Mh\text{-}1at}$ resistance gene with other resistance genes from other Solanum species. This is however only profitable when the resistance genes are complementary to the matching virulence in the nematode population. The detection of closer linked markers and subsequently designing closely linked CAPS markers will open the possibility to combine these different resistance genes in potato. Besides producing an assumable higher yield, resistant varieties can establish a reduction of residual population density that subsequently will cause less damage to the next crop in rotation. Further research should be carried out to assess differential interaction and synergic effects to a wide range of different M. hapla populations.

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Chapter 5

Analysis of inter-specific and intra-specific crosses to localise the Solanum fendleri derived resistance gene $R_{Mc1\text{-}fen}$ against M. chitwoodi and M. fallax on potato chromosome 11

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Abstract

An inter-specific and an intra-specific mapping population of the tetraploid species S. fendleri were developed for the genetic localisation of the gene $R_{Mc1-fen}$, conferring resistance to Meloidogyne chitwoodi and M. fallax. Results of the bio assay allowed a discrete classification of resistant and susceptible phenotypes. The resistance to both nematode species was in both mapping populations absolutely correlated. With Bulked Segregant Analysis a linkage group could be formed with AFLP markers comprising the $R_{Mc1-fen}$ locus. The use of a simple single locus specific PCR marker assay derived from the conversion of a relatively large AFLP fragment, in a reference population made it possible to align the linkage group to chromosome 11. The resistance gene mapped on a distal position of the long arm of chromosome 11, a region that is known to contain many resistance genes. The newly developed primer pair, however, could not distinguish the resistant and susceptible alleles in the mapping population. Further sequence analysis is necessary to regain this discrimination and to develop primers to use in marker assisted selection. The differences between the intra-specific and inter-specific mapping populations are discussed.

Introduction

Nematode species are important world-wide pests of potato. Various options for controlling and reducing their damage include the use of nematicides, deep ploughing, crop rotation and resistant cultivars. The growing of resistant cultivars is the most desirable and effective method of control. Due to substantial breeding efforts a large number of cultivars are currently available with adequate levels of resistance against the potato cyst nematode species Globodera rostochiensis Stone and G. pallida Woll.. The cultivation of these potato cyst nematode resistant cultivars allowed a decrease of the application of nematicides, but resulted in an increase of other nematode species, including the root-knot nematodes Meloidogyne chitwoodi Golden and M. fallax Karssen. These nematodes are of growing concern on the sandy and sandy-peat soils in the Netherlands (Molendijk & Mulder 1996). In the pacific North-West of the United States the root-knot nematode M. chitwoodi is already an important pest (Evans & Trudgill 1992). Recently it has been discovered in Texas as well (Szalanski et al. 2001). The broad host range of the Meloidogyne spp. impedes the control by means of crop rotation. Therefore the development of resistant cultivars is highly desirable. Unfortunately, no resistance to M. chitwoodi and M. fallax has been found in commercial potato cultivars (Janssen et al. 1995), but screening several wild Solanum species revealed resistance to these nematodes (Janssen et al. 1996a; Brown et al. 1988, 1991). One of these resistance genes, the R_{Mc1} locus, originating from S. bulbocastanum (2n=2x=24), has been mapped on chromosome 11 (Brown et al. 1996; Rouppe van der Voort et al. 1999). In the wild species S. fendleri (2n=4x=48) the gene R_{Mc2} was found, which provides resistance to both M. chitwoodi and M. fallax (Janssen et al. 1997b).

The rapid introgression of resistance genes and efficient selection of resistant offspring in commercial breeding programs would greatly benefit from indirect selection with molecular markers. Bulked Segregant Analysis (BSA)(Michelmore et al. 1991) allows a targeted

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identification of molecular markers with close linkage to the trait locus while minimising experimental work. However, BSA was originally designed for disomic inheritance. BSA will loose much efficiency when applied to tetraploid organisms, because simplex/nulliplex (Aaaa × aaaa) markers are required, with the A-allele being linked in coupling phase with the simplex target allele. Li et al. (1998) deduced (assuming that 25 % of the bands in AFLP fingerprints show a polymorphism), that the expected proportion of simplex/nulliplex markers is approximately 5 %. Only a quarter of the simplex/nulliplex markers will have the correct linkage phase.

Two different strategies can be pursued to generate a mapping population for the localisation of a wild species derived resistance gene. The first option is directed towards the identification of a resistant and a susceptible genotype within or between wild species gene-bank accessions. Next, these genotypes are crossed and the intra-specific offspring is evaluated to test for segregation of the target gene. Depending on the zygosity of the target locus and the ploidy level of the species, further backcrosses may be required to find a convenient segregation ratio. The level of polymorphism could be lower in the intra-specific mapping population, because S. fendleri is a self-pollinator, so an elevated level of homozygosity is expected.

The alternative option is introgression of the gene into the cultivated species by recurrent backcrosses, while screening for the presence of resistance. A commonly observed drawback of inter-specific introgression material is the reduced level of recombination between the introgression segment and the recipient genome (De Vincente & Tanksley 1991; Ganal & Tanksley 1996). Inter-specific backcrosses can be complex because of crossing barriers, and it will take several generations. However, this is not a waste of time, because introgression is also a goal on its own. Moreover, it will automatically change the original zygosity of the target gene and markers into the simplex/nulliplex condition required for BSA.

In this paper we compare both the intra-specific and the inter-specific approach and evaluate both systems on their merits. The level of polymorphism is compared between the intra- and inter-specific mapping population. In addition we will evaluate the level of recombination in the intra- and inter-specific mapping population, and utilize high levels of recombination to obtain the resolution required for map based cloning.

Materials & Methods

Plant material

Two different mapping populations were developed for the genetic localisation of the root-knot nematode resistance gene of S. fendleri (Janssen et al. 1997b; Brown et al. 1999). The design and parental genotypes of the inter-specific and intra-specific mapping populations are presented in Figure 1. The inter-specific BC_2 population RH4X-088 and the intra-specific BC_1 population M95-236 consisted of 121 and 83 offspring genotypes respectively.

The tetraploid S. fendleri genotypes 93-114-3 and 93-114-11 are the donors of resistance in the mapping populations. They originated as seedlings from the S. fendleri gene-bank accession BGRC8083 and were previously tested by Janssen et al. (1997b). On the basis of previous

research (unpublished results), the resistant genotypes M94-51-1 and RH4X-036-11 were expected to be simplex (Rrrr). Consequently both mapping populations are expected to segregate in a 1:1 ratio.

The seeds of the mapping population RH4X-088 were sown in vitro, and cuttings were multiplied on MS30 medium. Three weeks old in vitro cuttings were transplanted in the greenhouse in 350 cm³ clay pots containing silver sand and slow release NPK fertilizer ('Osmocote', Sierra Chemical Company, Milpitas, USA). During the first six days the relative humidity was kept near 100%. The temperature in the greenhouse was maintained at 20° C, with fluctuations of 4° The seeds of mapping population M95-236 were sown in soil, andC. cuttings were made for the resistance tests. Further conditions were similar to the RH4X-088 population. The mapping populations were tested in a completely randomised design with 3 to 4 replicates of each clone.

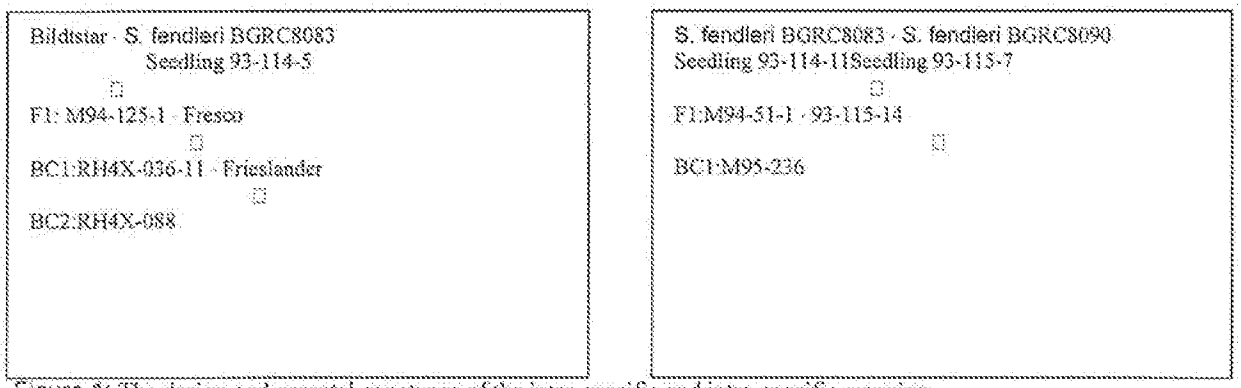


Figure 1: The design and parental genotypes of the inter-specific and intra-specific mapping populations

Meloidogyne resistance test

Four weeks after transplanting, the plants were inoculated with 1 ml of a water suspension containing approximately 500 juvenile (J2) larvae. All plants were tested for resistance against M. chitwoodi population "Co" (formally known as "CHB", Janssen et al. 1998) and M. fallax population "Fa" (formally known as "CHB", Janssen et al. 1998). Eight weeks after inoculation the root systems were rinsed and stained with Phloxine-B (Dickson & Struble 1965). The number of egg masses on resistant and susceptible clones is ranging from 0-11 and 25-250, respectively. This allows a discrete classification of resistant and susceptible phenotypes on the basis of the number of egg masses. Plants with a poorly developed root system were omitted from further analysis.

Marker analysis

Total genomic DNA was extracted from leaf material as described by van der Beek et al. (1992). The AFLP technique was performed according to Vos et al. (1995). The AFLP template was prepared using the enzyme combinations EcoRI/MseI and PstI/MseI. Nomenclature of the AFLP markers is based on the template enzyme combination abbreviated to one letter, followed

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by letters that represent the selective nucleotides and a number describing the visually estimated mobility of the fragment relative to the bands of the SequaMark 10 base ladder (Research Genetics, Huntsville, AL, USA). BSA was performed by pooling equal aliquots of secondary template of eight random chosen resistant and eight random chosen susceptible plants of the BC_2 population RH4X-088. For the intra-specific population M95-236, the bulks consisted of 10 plants. Usually the two parents are included during a BSA experiment to allow detection of markers in coupling as well as in repulsion phase. At the tetraploid level, linked markers in repulsion will not show bulk specificity and therefore inclusion of parental samples in BSA was irrelevant. The inter-specific mapping population RH4X-088 was tested with 44 primer combinations. The intra-specific population M95-236 however, was screened with 304 of the 512 possible primer combinations of Eco+ANN and Mse+ CNN.

AFLP based on PstI/MseI

template was applied on the RH4X-088 population only. In this second round of screening, two susceptible and one resistant bulk were screened with all 256 combinations of 16 M+CNN primers combined with 16 P+NN primers. AFLP primer combinations with a bulk specific amplification product, indicating a putatively linked marker, were verified on the individual genotypes of the bulks. Only those primer combinations without a recombinant in these individuals were applied on the complete mapping population. The chromosome number of the linkage group was confirmed by testing the chromosome 11 specific CAPS marker M39b (Brigneti et al. 1997) using the PCR conditions as described by the authors. The M39b PCR products were digested with the restriction enzyme DdeI and separated on a 1.5 % agarose gel. The genetic data of the marker loci and phenotypic resistance data were analysed using Joinmap version 2.0 (Stam & van Ooijen 1995).

Conversion of the AFLP marker into a simple single locus specific PCR marker assay.

For the conversion of AFLP markers into a simple marker assay, AFLP bands were excised from dried polyacrylamide gels and diluted in 100 µl of TE for 1 h at room temperature. The excised AFLP fragments were re-amplified using elongated AFLP primers. These M13 AFLP primers have an extra stretch of nucleotides at the 3' end, complementary to the M13 primer site, to allow the sequencing of the PCR products with standard M13 sequencing primers. Each band was sequenced at least twice. When possible, a primer pair was developed with an annealing temperature of at least 45° Primers with a lower annealing temperature are moreC. sensitive to mismatch and will consequently produce additional non-specific products.

The ultra dense map of potato (Isidore et al. 2003; http//:www.dpw.wur.nl/pv/aflp/catalog.htm) was used as a reference population to enable the alignment of the linkage group to the correct potato chromosomes.

Results

Inheritance of resistance to M. chitwoodi and M. fallax

Most genotypes of the mapping population, that were evaluated for resistance to M. chitwoodi and M. fallax, could be clearly classified as resistant or susceptible (Table 1). Occasionally some egg masses were formed on resistant genotypes but never more than 11, whereas in the susceptible genotypes over 200 egg masses could be found. The Chi-square test showed that the segregation of resistant and susceptible phenotypes did not deviated from a 1:1 ratio in the intra-specific population M95-236 (χ^2=1.39; p=0.24). A significant deviation from a 1:1 segregation ratio (χ^2=5.83; p=0.016) was observed in population RH4X-088 where an excess of susceptible genotypes occured. The segregation of the resistance to both nematode species M. chitwoodi and M. fallax was absolutely correlated.

Table1: Number of resistant, susceptible and unknown genotypes of the inter-specific mapping population RH4X-088 and intra-specific mapping population M95-236.

	Resistant	Susceptible	Unknown
RH4X-088	45	71	3
M95-236	41	31	1

AFLP analysis

Resistant and susceptible bulks of the intra-specific population M95-236 were screened with 304 Eco/Mse AFLP primer combinations, and only one AFLP marker with linkage to the resistance gene was obtained. The 44 Eco/Mse primer combinations that were tested in the inter-specific population RH4X-088 resulted in eight bulk specific amplification products. When tested on the complete offspring, four AFLP bands appeared to be false positives. The remaining four AFLP markers showed significant linkage with the locus involved in resistance and had a similarly skewed segregation pattern. The second round of BSA of population RH4X-088 with 256 Pst/Mse primer combinations resulted in 15 additional bulk specific bands. Seven of these primer combinations showed AFLP markers without recombination in the subset of 16 genotypes of the bulks. These seven primer combinations were tested on the complete offspring and tight linkage was confirmed between these seven markers and the resistance locus.

The marker E+ACT/M+AAC_85 was the only marker that was obtained in the intra-specific population. The marker mapped at a distance of 2.5 cM from the resistance locus. In the inter-specific map this marker was not observed using BSA, because another AFLP-fragment from one of the susceptible S. tuberosum parents with almost the same mobility was segregating. The presence of this S. tuberosum derived AFLP band overshadowed the presence or absence of the S. fendleri derived AFLP band that was linked to the resistance locus. Marker E+ACT/M+AAC_85 was scored afterwards in the inter-specific population, in those individuals that did not have the S. tuberosum derived AFLP fragment. The estimated genetic distance between the resistance locus and this marker was 2 cM. None of the ten AFLP markers linked with the resistance locus in the inter-specific population was polymorphic in the intra-specific population.

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Genomic localisation of the resistance locus

To identify the chromosome number of the linkage group, three AFLP fragments were excised from gel and sequenced to allow the development of locus specific PCR primers. Only one primer pair, developed on the sequence of the AFLP marker P+GG/M+CAC_302, amplified a marker that was polymorphic in the reference mapping population SH×RH (Isidore et al. 2003). The PCR products gave polymorphic bands after digestion with restriction enzyme MboI, resulting in two bands of approximately 200 and 150 bp (Figure 2). Parent SH had both bands ($A_{200}A_{150}$) while parent RH only had the lower band (A_{150}). To explain the segregation of 76 offspring genotypes, the following model was most plausible.

$A_{200}A_{150} \times A_{150}A_{null} = ¼\ A_{200}A_{150} + ¼\ A_{200}A_{null} + ¼\ A_{150}A_{null} + ¼\ A_{150}A_{150}$, of which the latter two can not be distinguished.

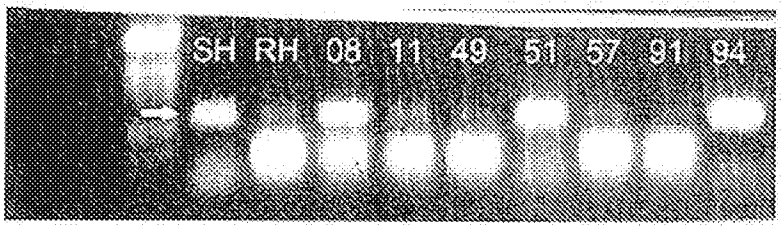


Figure 2: Part of the 1.5 % agarose gel with the PCR product with different segregation types in the mapping population SH×RH, obtained with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302, after digestion with restriction enzyme MboI. The segregating PCR product is indicated with an arrow.

The expected ratio was 1:1:2 and the observed ratio 22:23:31 fitted this model (χ^2=2.61, p=0.27). The A_{200} allele could be mapped in bin SH11_05 at a distal position on the long arm of chromosome 11. Therefor, in reverse the chromosome number of the linkage group of population RH4X-088 comprising the resistance locus was confirmed by testing the chromosome 11-specific CAPS marker M39b (Figure 3, Brigneti et al. 1997).

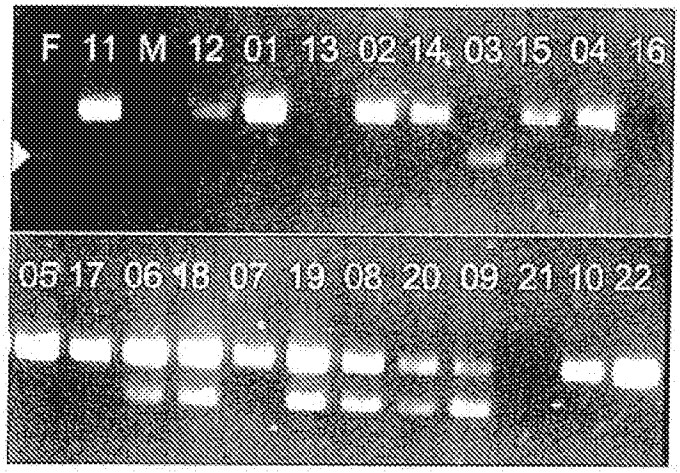


Figure 3: Part of the 1.5 % agarose gel with the PCR product segregating in the inter-specific mapping population, obtained with the M39b primerpair. The mapped PCR product is indicated with a triangle.

In the progeny no recombinants were detected between the M39b marker and the resistance locus. The construction of a linkage group of the inter-specific population, comprising eleven AFLP markers, M39b and the resistance locus resulted in a map with a total length of 11 cM (Figure 4). According to the proposed nomenclature for R genes (Chapter 6), we will name the resistance gene $R_{Mc1\text{-}fan}$.

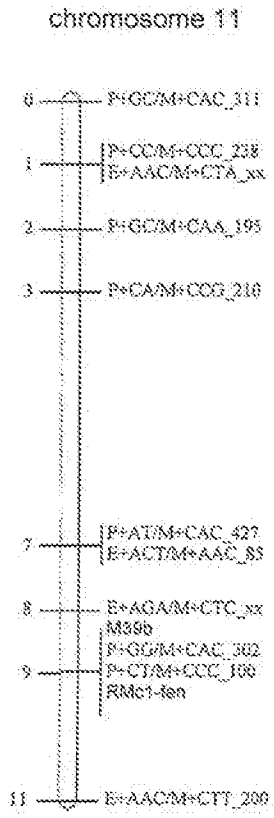


Figure 4: Position of the $R_{Mc1\text{-}fan}$ locus on the long arm of chromosome 11 of the resistant clone RH4X-088. Chromosome orientation is according to Dong et al. (2000). AFLP markers of which the sizes are not estimated are marked by xx.

Amplification products derived from a PCR with the primers based on the sequence of AFLP marker P+GG/M+CAC_302, were also segregating in the mapping population (Figure 5). This primer pair gives one band of approximately 100 bp in the resistant parent of the inter-specific population and one band with the size of approximately 200 bp in the susceptible parent. In the mapping population the marker segregates in four phenotypic classes with: both bands, one band of the resistant parent, one band of the susceptible parent or without amplification of a band. In combination with the phenotypic data the progeny could be divided in six classes (Table 2).

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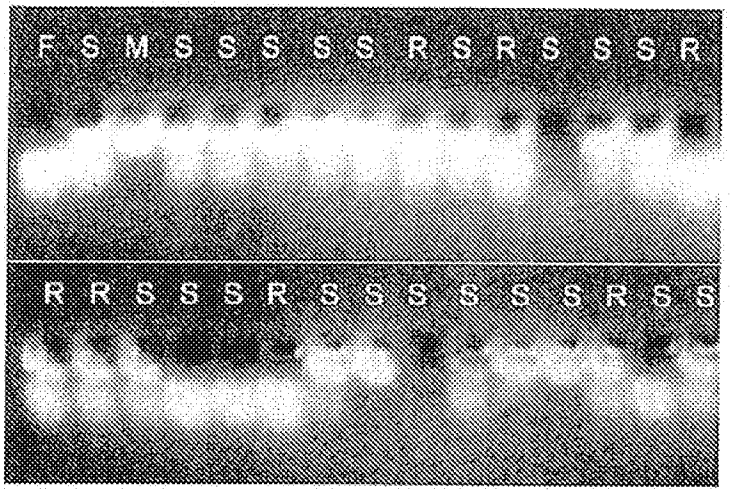


Figure 6: Parts of the 1.5 % agarose gel with the PCR product with different segregation types in mapping population RH4X-088, obtained with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302. The resistant female parent RH4X-036-11 is indicated with a F, the susceptible male parent Friestander is indicated with a M. The resistant and susceptible progenies are indicated with respectively a R and a S.

Table 2: number of progeny per distinguished classe with the primer pair based on the sequence of AFLP marker P+GG/M+CAC_302, and the phenotypic data.

Phenotypic data	Amplification product	number of progeny
Resistant	100 bp, 200 bp	24
Resistant	100 bp	14
Susceptible	100 bp, 200 bp	32
Susceptible	200 bp	8
Susceptible	100 bp	11
Susceptible	No band amplified	2

Discussion

In this study, two different strategies have been compared to localise a gene involved in resistance to the root-knot nematode species M. fallax and M. chitwoodi. One striking difference was the level of polymorphism between the two populations. Only one AFLP marker was detected in the intra-specific mapping population. The low level of within-species polymorphisms, as reflected by the low number of bulk-specific AFLP markers, is clearly the result of the reproduction mode of S. fendleri. As a rule of thumb self-fertilising species show a lower level of genetic polymorphisms. On the other hand the genetic dissimilarity between the wild species introgression segments and the recurrent potato parents may have caused an elevated level of polymorphic markers in the BC_3 inter-specific mapping population.

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Evaluation of the assumed reduction of the level of recombination in inter-specific hybrids is somewhat complicated, because the intra-specific map is composed of only one marker and the $R_{Mc1\text{-}fax}$ locus. The intra-specific map distance of 2.5 cM is slightly larger than the distance of 2 cM in the inter-specific map. Although in addition three markers of the inter-specific map cluster without recombination with the R-gene, we feel that the amount of data is inadequate to conclude that the results render to support for reduction in the level of recombination.

Most relevant from the breeder's point of view is the following. The use of inter-specific mapping populations allows the parallel development of a mapping population, with the development of resistant breeding lines. In addition, markers that show close linkage in experimental material can be used in breeder's material without validation. Finally, the marker data allowed the selection of genotypes RH4X-088-50 and RH4X-088-87 using marker P+AT/M+CAC_427 which has a recombination event adjacent to the $R_{Mc1\text{-}fax}$ locus, thus minimising wild species linkage drag.

Our data confirm the monogenic resistance to the two nematode species, as suggested by Janssen et al. (1997b). In the same paper Janssen et al. showed that the resistance to M. fallax and M. chitwoodi is correlated, as is confirmed by our results in both mapping populations. This correlation could mean that the resistance gene is involved in recognition of a similar avirulence gene product from both M. chitwoodi and M. fallax. It is not unlikely for the two nematode species, being very close related, to have similar avirulence gene products. Rossi et al. (1998) showed that the nematode resistance gene Mi of tomato also confers resistance to the very distinct related potato aphid. Apparently the avirulence gene products of these two organisms, which belong to different phyla, are very similar. On the other hand, it is not unlikely that avirulence genes of M. chitwoodi and M. fallax differ. Besides isozyme band pattern, the two species are also distinguished by their differential host ranges, which might be due to a different set of avirulence genes. It is shown that maize is a non-host for M. fallax whereas it is a good host for M. chitwoodi (Van Meggelen et al. 1994). In the case that M. chitwoodi and M. fallax really differ in their set of avirulence genes, the correlated resistance to both nematode species could be based on different R-genes that are members of the same resistance gene cluster. The cloning of the $R_{Mc1\text{-}fax}$ resistance gene in conjunction with a complementation assay will provide the opportunity to find out if the same gene regulates resistance to both nematode species, or that two different genes are present in the same cluster.

Occasionally some egg masses were formed on resistant genotypes of either mapping population. In a greenhouse experiment, the selection of virulence in M. chitwoodi towards the $R_{Mc1\text{-}fax}$ gene was easily achieved (Janssen et al. 1998). The nematode population used in our study is a field population with an unknown composition of virulent and avirulent genotypes. Considering the unequivocal difference between resistant and susceptible plants, the fraction of resistance breaking nematodes is not high. Most likely one growing season with a $R_{Mc1\text{-}fax}$ resistant potato cultivar will impose a strong selection, leading to an increasing proportion of virulent genotypes. However the current absence of any resistance in potato necessitates the application of the $R_{Mc1\text{-}fax}$ gene, although the selection of virulence by resistant cultivars should be monitored.

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We successfully converted an AFLP marker into a single locus PCR marker. With this converted primer pair three alleles could be distinguished by a band of 200 bp, a band of 100 bp and no band. The genetic model of the resistant parent of the inter-specific population is ×vxvL L ll, where L is the resistant allele showing a band of 100 bp and L is the susceptible allele ×vshowing the same 100 bp band. Because the resistant and susceptible alleles L and L cannot be distinguished, this marker can not be used for marker assisted selection. Further sequence analysis is necessary to regain the discrimination between the susceptible and resistant alleles. Therefore we will sequence the flanking DNA of the AFLP marker and subsequently construct a SNP based on the discriminating basepairs of the resistant and susceptible genotypes. Because we did not detect a recombination event between the converted AFLP marker and the resistance gene, the SNP developed on the flanking DNA will be useful for marker assisted introgression of the resistance in potato.

The markers detected with BSA represented alleles from S. fendleri origin and did not coincide with AFLPs that have been observed in earlier studies. Therefore it was not possible to align the linkage group containing the $R_{Mc1\text{-}fen}$ locus with existing AFLP linkage maps. If co-migrating alleles from S. tuberosum origin would have been observed, from which the map position was known, this would have provided an indication for the chromosomal position of this linkage group (Rouppe van der Voort et al. 1997a). Previous research showed the failure of using this strategy to align S. bulbocastanum alleles to the S. tuberosum linkage map. This was probably due to the distant relation of the two species (Rouppe van der Voort et al. 1999). Although S. fendleri, in contrast to S. bulbocastanum, is cross compatible with S. tuberosum, the genetic distance to S. tuberosum is apparently also too large to allign linkage maps by means of common AFLP markers.

Based on the sequence of a relative large AFLP fragment (302 bp), we were able to develop a primer pair, that gave in a diploid potato population (SH×RH) rise to segregating PCR products. With segregation analysis the linkage group could be aligned to chromosome 11, which could be confirmed by the chromosome 11 specific CAPS marker M39b. According to Dong et al. (2000) the physical orientation of chromosome 11 has an opposite orientation to the current potato genetic linkage group. The RFLP marker GP125 is localised on the long arm of chromosome 11, but in current linkage groups on the north arm. The marker M39b is linked at the distal side to GP125. The resistance gene $R_{Mc1\text{-}fen}$ should therefore be mapped on a distal position on the long south arm of chromosome 11. This is a region that is known to contain many resistance genes. In the same region the $R_{Mc1\text{-}blb}$ gene is mapped, a resistance gene of S. bulbocastanum which gives also resistance to M. chitwoodi and M. fallax (Brown et al. 1996; Rouppe van der Voort et al. 1999). It is common feature that resistance genes are clustered in potato. Resistance genes like R3, R6 and R7, which give resistance to different races of Phytophthora infestans, are located next to each other in a narrow genome segment (El-Kharbotly et al. 1994, 1996). The found locus $R_{Mc1\text{-}fen}$ coincides with a resistance gene cluster containing genes of related and non-related pathogens. Besides the resistance gene $R_{Mc1\text{-}blb}$, the potato cyst nematode resistance gene Gpa3 (Wolters et al. 1998), the wart disease resistance gene Sen1 (Hehl et al. 1999), the virus resistance genes Ry_{sto} (Hämäläinen et al. 1997), Ra_{adg} (Hämäläinen et al. 1998) and Ry_{sto} (Brigneti et al. 1997), the potato cyst nematode

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resistance QTL Gro1.3 (Kreike et al. 1993) and the potato leafroll virus resistance QTL PLRV.1 (Marczewski et al. 2001), are localised on this distal part of chromosome 11. Other resistance gene clusters in potato are found on chromosome 5 and 12 (Gebhardt & Valkonen 2001). Most of the cloned resistance genes contain similar components involved in the recognition system of the plant. Based on this homology, it is hypothesised that the genes are evolutionary related and restricted to a few loci on the genome. The clustered resistance genes may be evolved by gene duplications followed by structural and functional diversification. This diversification can lead to gene clusters containing homologous genes acting to distinct pathogen species, like the two resistance genes of the gene cluster on chromosome 12, that give resistance towards potato virus X and the potato cyst nematode G. pallida (Van der Vossen et al. 2000). It is therefore not unlikely that the mapped $R_{Mcf-fax}$ gene is homologous to other resistance genes in the cluster on chromosome 11.

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Chapter 6

Mapping and comparative analysis of the chromosome 11 root-knot nematode (Meloidogyne chitwoodi) resistance gene $R_{Mc1\text{-}hou}$ from wild potato (Solanum hougasii).

Jan Draaistra, Ronald C.B. Hutten, Ruth van der Heide, Jessie Smeets, Herman J. van Eck

Abstract

The resistance to the root-knot nematodes Meloidogyne chitwoodi and M. fallax present in the hexaploid wild potato species Solanum hougasii was efficiently mapped using bulked segregant analysis (BSA) in an inter-specific mapping population. The BSA, carried out with 394 AFLP primer combinations, resulted in a linkage group consisting of the resistance locus and 14 AFLP markers representing a genetic interval of 24 cM. The monogenic resistance locus $R_{Mc1\text{-}hou}$ is located at the distal end on the long arm of chromosome 11, a well known hotspot of resistance genes, among others the root-knot nematode resistance genes $R_{Mc1\text{-}blb}$ and $R_{Mc1\text{-}fen}$. The effect of the resistance gene introgressed from S. hougasii was compared with the other two mapped root-knot nematode resistance genes from S. bulbocastanum and S. fendleri. A comparative analysis of virulence and resistance was performed with Solanum genotypes carrying an R_{Mc1} gene and six nematode populations of M. chitwoodi. The tested nematode populations represented avirulent isolates from natural populations of race 1 and 2, a resistance breaking nematode population of race 2 formerly known as race 3, and a mono-female line selected from a successful infection event on S. fendleri by an avirulent population of race 1. The resistance breaking capacity of two virulent populations is confirmed. In the avirulent populations a low frequency of successful infection events is observed. The genotypes carrying an R-gene do not show clear differential interaction patterns with the nematode populations, suggesting a similar spectrum of resistance for $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}blb}$, and $R_{Mc1\text{-}fen}$.

Introduction

The potato (Solanum tuberosum L.) is like most modern crop plants vulnerable to a range of pests and diseases. One of the pests causing considerable losses in yield and quality is the Colombian root-knot nematode Meloidogyne chitwoodi (Golden et al. 1980). The nematode is found in different locations throughout the western states of the USA (Evans & Trudgill 1992, Santo et al 1980, Szalanski et al 2001), Mexico, Portugal, South Africa, the Netherlands and Belgium (summarized in Van der Beek 1997). This nematode has a broad host range and is therefore difficult to control via crop rotation. Breeding for resistance is considered to be an effective and environmentally safe strategy to control this nematode.

Within the cultivated potato gene pool only susceptible genotypes are found. Several wild Solanum species have been identified as valuable sources for the introgression of resistance genes. Among these are S. bulbocastanum Dun., S. hougasii Corr. and S. fendleri A.Gray, of which the resistance genes perform as a monogenic and dominant trait (Brown et al. 1996, 1999; Janssen et al. 1998; Rouppe van der Voort et al. 1999). So far, two resistance genes have been mapped: $R_{Mc1\text{-}blb}$ of S. bulbocastanum (Brown et al. 1996, Rouppe van der Voort et al. 1999), and $R_{Mc1\text{-}fen}$ of S. fendleri (Chapter 5). Both resistance genes mapped on a similar distal map position on the long arm of chromosome 11. The resistance genes in these species share, besides their map position, also another feature. Both appeared to be effective to the false root-knot nematode M. fallax (Janssen et al. 1997c, Rouppe van der Voort et al. 1999, Chapter 5). M. fallax is a species genetically closely related to M. chitwoodi (Karssen 1996).

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Besides the Netherlands, the presence of this nematode species is also documented in France (Daher et al. 1996) and New Zealand (Marshall et al. 2001).

At present two races of M. chitwoodi are recognised. The basic difference between race 1 and race 2 resides in their reproduction on the alfalfa cv. Thor and the carrot cv. Red Cored Chantenay. M. chitwoodi race 2 reproduces on alfalfa but not on carrot. Conversely, alfalfa is a poor host and carrots are suitable for M. chitwoodi race 1 (Santo & Plokterton 1985, Mojtahedi et al. 1988). A virulent biotype of race 2, previously separately indicated as a race 3, is able to overcome the resistance in S. bulbocastanum (Mojtahedi et al. 1994). In a study with eight different M. chitwoodi populations of race 1 and five Solanum species, no significant interaction was found between the plant genotypes and nematode populations, suggesting little variation in virulence factors across sampled populations of race 1 (Van der Beek et al. 1998b). However, within each population multiple virulence factors could be present at variable allele frequencies. In a greenhouse experiment Janssen et al. (1998) succeeded in the selection of mono-female M. chitwoodi lines with increased virulence that could overcome the resistance gene $R_{Mc1\text{-}den}$ by multiplying egg-masses which are occasionally formed on a resistant genotype. Most likely this virulence factor was already present at a low allele frequency in the largely avirulent population.

In this paper we describe the genetic localisation of the resistance gene $R_{Mc1\text{-}hou}$ present in S. hougasii. In addition, the spectrum of this resistance gene was analysed using a series of isolates of M. chitwoodi. Finally, the resistance spectrum of the current $R_{Mc1\text{-}hou}$ is compared with the resistance genes $R_{Mc1\text{-}blb}$ and $R_{Mc1\text{-}den}$ to allow discussion on the value of these genes in breeding.

Materials & Methods

Plant material

The pedigree of the mapping population is presented in Figure 1. The genotype M94-110-2 is a pentaploid inter-specific hybrid from a cross between the hexaploid S. hougasii BGRC55203 and a tetraploid S. tuberosum breeding line. This S. hougasii genotype is resistant to M. chitwoodi and M. fallax (Janssen et al. 1997c). Backcrosses were made to assure segregation and a simplex inheritance of monogenic resistance. On the basis of its high resistance and agronomic value the BC1 clone RH4X-029-2 was chosen as the resistant parent for a BC2 mapping population.

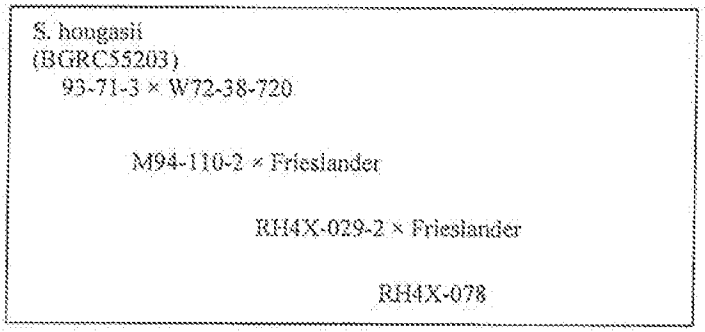


Figure 1. Crossing scheme of the mapping population RH4X-078

Seeds of the RH4X-078 population were sown in vitro on a MS30 medium (Murashige & Skoogе 1962). After germination, 111 seedlings were multiplied in vitro to obtain a sufficient number of plants for replicated resistance assays, DNA extraction, and genotype maintenance. In vitro grown parents of the mapping population and the susceptible control variety Bintje were included in the resistance test. Three weeks after sub-culturing the plant material, the rooted cuttings were transplanted in 350 cm^3 clay pots containing silver sand and slow release NPK fertiliser ('Osmocote', Sierra Chemical Company, Milpitas, USA). The pots were placed in a greenhouse in a completely randomised design with initially 4 replicates of each clone. The temperature in the greenhouse ranged between 18° and 25° During the first six days afterC. transplanting the relative humidity was kept near 100%. Four weeks after transplanting, the plants grew vigorously, and were inoculated with 1 ml of a water suspension containing approximately 500 juvenile (J2) larvae. All plants were tested for their level of resistance against M. chitwoodi population Co (formally known as CHE, Janssen et al. 1998) and M. fallax Fa (formally known as CHB, Janssen et al. 1998). Eight weeks after inoculation, the root systems of the plants were harvested, and rinsed to remove the sand, and stained with Phloxine-B (Dickson & Struble 1965). The clear difference between the amount of egg masses on resistant and susceptible genotypes allowed classification of the offspring genotypes. Besides of this classification, the number of egg-masses formed on the parents, controls and resistant plants were counted. The size of the root systems was recorded and plants with a small root system were excluded from further analysis.

Molecular analysis

Three in vitro cuttings per genotype were used for maintenance and DNA isolation. DNA isolation was performed as described by Van der Beek et al. (1992). The AFLP technology was carried out basically as described by Vos et al. (1995). Nomenclature of the AFLP-markers is based on the template enzyme combination abbreviated to one letter, followed by letters that represent the selective nucleotides, and a number describing the visually estimated mobility of the fragment relative to the bands of the SequaMark 10 base ladder (Research Genetics, Huntsville, AL, USA). The Bulked Segregant Analysis (BSA) method (Michelmore et al. 1991) was applied to identify linked markers. A set of eight susceptible plants and a set of eight resistant plants were used to compose the susceptible and resistant bulk. For this purpose we mixed equal aliquots of secondary template (restriction-ligation product amplified with pre-selective primers or E+A, M+C). BSA-AFLP was performed using 138 combinations of EcoRI+ANN primers with MseI+NNN primers. A second round of BSA was performed using all 16 PstI+NN - 16 MseI+CNN primer combinations. These 256 primer combinations were performed on one resistant bulk and two susceptible bulks, each comprising eight individuals. Primer combinations showing bulk specific fragments were verified on separate individuals of the entire mapping population (or a subset) to confirm genetic linkage with the resistance gene. The genetic data were analysed using JoinMap version 2.0 (Stam & Van Ooijen, 1995).

In order to assign the correct chromosome number to the linkage group, the strategy of Rouppe van der Voort et al. (1997b) was followed. This strategy implies that AFLP markers with equal mobility on gel, are most likely to show DNA sequence homology and represent the same locus

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on a linkage map. Therefore the reference genotypes C, E, AM, RH and SH were also included during analyses of the primer combinations that showed a bulk specific AFLP marker. In the case co-migrating markers were present in any reference genotype, the map position of the marker could be retrieved from a database (Rouppe van der Voort et al. 1998a; http//:www.dpw.wur.nl/pw/aflp/catalog.htm). To confirm the identity of the linkage group, the CAPS marker M39b (Figure 2) was analysed using PCR-conditions according to Brigneti et al. (1997). The PCR-product was digested with the restriction enzyme DdeI (Brigneti et al. 1997).

Comparative analysis of virulence and resistance spectrum

The effect of the resistance gene introgressed from S. hougasii was compared with resistance genes from S. bulbocastanum and S. fendleri. Plant material carrying the resistance genes from the three potato species was analysed with six nematode populations of M. chitwoodi listed in Table 1. The populations Ca, Cat, Cba and Cbd represent natural populations that were propagated without selection. The isolate Cbh is a resistance breaking nematode population, which was formerly known as race 3 (Mojtahedi et al. 1994). The isolate Cel represents a mono-female line that was selected from a successful infection event on S. fendleri by the avirulent population Co (formerly known as population CHE; Janssen et al. 1998). The bioassay was performed in 4 replications as described above, but the amount of newly formed egg-masses was counted to allow statistical analysis of the interactions between nematode populations and potato genotypes. The potato genotypes used for this comparative analysis were the BC3-plants 95-B3-02, 95-B3-03 and 95-B3-13R derived from S. bulbocastanum by a cross between 398.89 - A84118.3 that was used to map the resistance gene $R_{Mcl\text{-}blb}$ on chromosome 11 (Rouppe van der Voort et al. 1999). An additional BC3-plant of S. bulbocastanum: 95-B5-07 was derived from a cross between 398.89 - Ranger Russet. The genotype M94-125-1 represents the resistant parent of a S. fendleri derived mapping population that was used to localise $R_{Mcl\text{-}fen}$ (Chapter 5). The resistant parent RH4X-029-2 of the S. hougasii mapping population, used in the present study, and the S. tuberosum cultivars Gloria (1963) and Bintje were added in the test as resistant and susceptible controls, respectively.

Table 1. Isolates of M. chitwoodi used in the virulence test.

Isolate	Race	Origin	Reference
Ca	1	NL	Van der Beek et al. (1999)
Cat	1	NL	Van der Beek et al. (1997)
Cba	1	USA	Van der Beek et al. (1999)
Cbd	2	USA	Van der Beek et al. (1999)
Cbh	2	USA	Van der Beek et al. (1999)
Cel	1	NL	Janssen et al. (1998)

[1] a resistance-breaking biotype which was formerly known as race 3 (Mojtahedi et al. 1994)
[2] virulent line from isolate Co (formerly known as CHE)

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Results

Resistance in the S. hougasii mapping population

The level of resistance across offspring genotypes did not fit a continuous distribution, but fell apart in two clearly distinguishable classes. Plants, which could be classified as resistant, showed between 0 and 4 newly formed egg-masses; on plants classified as susceptible the estimated amount of egg-masses ranged from 40 to 350. The high amount of egg-masses that developed on the susceptible progeny was similar to the amount of egg-masses formed on the susceptible control Bintje. Because of a poorly developed root system 7.1 % of the tested plants were excluded for further analysis. The nematode assay revealed a clear segregation of 56 susceptible and 53 resistant plants in mapping population RH4X-078, fitting the 1:1 Mendelian ratio (χ^2_{adj} 0.08) of a monogenic trait. Therefore we conclude the presence of a monogenic resistance locus, named $R_{Mcf\text{-}hou}$, according to the gene nomenclature proposed by Brown et al. (1999). The average number of egg-masses formed by M. chitwoodi and M. fallax on the parents, on the susceptible control and on the resistant progeny is listed in Table 2. The maximum number of egg-masses that was observed on resistant clones was four and three egg-masses by M. chitwoodi and M. fallax, respectively. The segregation of the resistance against M. chitwoodi and M. fallax was completely correlated.

Table 2. Mean amount of egg-masses of M. chitwoodi and M. fallax found on the parents of the mapping populations, the resistant genotypes of the mapping population and on the susceptible control Bintje.

	RH4X-029-02	Friesland	Resistant progeny	Bintje
M. chitwoodi	0.3	23.3	0.2	108.3
M. fallax	0.0	27.0	0.1	105.3

AFLP-analysis of the S. hougasii mapping population

Resistant and susceptible bulks were composed from eight offspring genotypes randomly chosen from the resistant and susceptible class. The parents and bulks were screened with a total of 394 AFLP primer combinations (138 EcoRI/MseI and 256 PstI/MseI). Bulk specific AFLP bands, showing an AFLP-fragment present in the resistant bulk only, were observed in 30 primer combinations. Bulk specificity was verified using the separate offspring templates. Fourteen of the thirty AFLP-fragments showed genetic linkage with resistance. This provides a further confirmation of the monogenic character of this resistance. The linkage map comprising these 14 AFLP markers represents a genetic interval of 24 cM (Figure 2).

The strategy of Rouppe van der Voort et al. (1997b) to determine the chromosomal identity of the linkage group was not successful. None of the scored AFLP-markers was present among the reference genotypes of the AFLP-catalogue (Rouppe van der Voort et al. 1998a). However one co-migrating AFLP marker was detected in another mapping population RH4X-088 (Chapter 5). The resistant parent RH4X-036-11 of this mapping population contains the $R_{Mcf\text{-}hou}$ resistance gene. The $R_{Mcf\text{-}hou}$ resistance gene was mapped at the distal position on the long arm of chromosome 11 and showed no recombination with the AFLP marker P+GG/M+CAC_302.

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This AFLP marker was the same as the marker that mapped most close to the resistance gene in the S. hougasii mapping population at a distance of 1 cM (one recombination event). This result demonstrated once more the locus-specificity of AFLP markers and the utility of co-migrating markers to detect the chromosomal identity of a linkage group. Independent confirmation was obtained with the chromosome 11 specific CAPS marker M39b, that maps to $R_{y\text{-}sto}$ and $R_{Mc1\text{-}blb}$ (Brigneti et al. 1997, Rouppe van der Voort et al. 1999). This CAPS marker mapped in the S. hougasii mapping population at a distance of 2 cM (two recombination events) from the $R_{Mc1\text{-}hou}$ resistance gene (Figure 2).

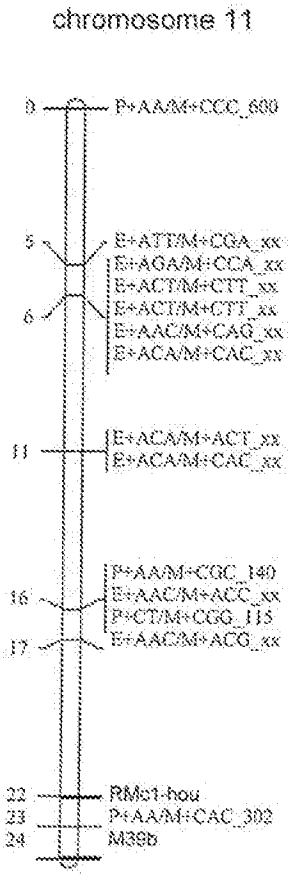


Figure 2: Position of the $R_{Mc1\text{-}hou}$ locus on the long arm of chromosome 11 of the resistant clone RH4X-078. Chromosome orientation is according to Dong et al. (2000). AFLP-markers of which the sizes are not estimated are marked by xx.

Comparative analysis of the resistance spectrum of R_{Mc1}-genes

Eight Solanum genotypes have been tested for their level of resistance against six root-knot nematode populations. The average number of the newly formed egg-masses observed in this assay is listed in Table 3. The large differences in the number of egg-masses provide clear evidence for the absence and presence of R-genes in potato genotypes, as well as the absence and presence of specific avirulence factor(s) in the nematode populations. The S. tuberosum clones do not carry effective R-genes, since large numbers of egg-masses are formed. The

genotypes that carry an R-gene do not show clear differential interaction patterns with the nematode populations, suggesting a similar spectrum of resistance for $R_{Mc1\text{-}bcu}$, $R_{Mc1\text{-}stb}$, and $R_{Mc1\text{-}ten}$.

The resistance breaking capacity of population Cbh and Ccl is confirmed. These populations produce egg-masses on material with an R-gene at a level that equals susceptible S. tuberosum. In the avirulent populations Ca, Cat, Cba and Cbd a low frequency of successful infection events is observed. For Cbd the rate of successful infections is considerably higher as compared with the avirulent populations Ca, Cat and Cba. Remarkably, the more virulent population Cbd did not produce egg masses on the genotypes 95-B3-03 and 95-B3-13R that carry $R_{Mc1\text{-}stb}$. Within the group of six resistant potato genotypes analysis of variance showed a significant effect of the potato genotype on the number of egg masses produced by Cbd ($p=0.019$). No significant effect of potato genotype on egg mass production was observed for other nematode populations (data not shown), because of the highly variable number of egg masses between replications and variation in root system development.

Table 3. Average number of newly formed egg-masses on eight Solanum genotypes using six different root-knot nematode populations.

Solanum spp. and genotype	R-gene	Root-knot nematode population: Ca race 1 NL	Cat race 1 NL	Cba race 2 USA	Cbd race 2 USA	Cbh [1] race 2 USA	Co (CHE) race 1 NL	Ccl [1] race 1 NL
S. tuberosum								
Bintje	None	51.8	140.0	135.5	134.5	110.0	susceptible	171.5
Gloria	None	100.8	75.8	24.3	49.0	109.3	susceptible	56.3
S. bulbocastanum								
95-B3-02								
95-B3-03	$R_{Mc1\text{-}stb}$	0.0	0.0	1.0	5.3	97.3	resistant	97.0
95-B3-13R	$R_{Mc1\text{-}stb}$	0.0	2.0	0.7	0.0	67.0	resistant	153.5
95-B5-07	$R_{Mc1\text{-}stb}$	0.0	0.0	0.3	0.0	90.8	resistant	123.8
S. fendleri	$R_{Mc1\text{-}stb}$	0.0	0.0	0.3	5.7	47.3	resistant	227.3
M94-125-1								
S. hougasii								
RH4X-29-2								
	$R_{Mc1\text{-}ten}$	0.3	0.0	0.3	15.0	84.0	resistant	103.5
	$R_{Mc1\text{-}bcu}$	0.0	0.0	1.0	5.0	82.5	resistant	28.5

1) The population Cbh (also known as CAMc2) was formerly regarded as an USA race 3 population because of its ability to multiply on a resistant S. bulbocastanum clone (Mojtahedi et al. 1994). Population Ccl represents a mono-female line that was selected from an occasional infection event on S. fendleri of population Co (Janssen et al. 1998).

2) The amount of inoculum of population Co that could be reared was insufficient to allow accurate testing. The results for Co were added for the sake of comparison, and are based on previous mapping experiments (see text). The resistant plants occasionally formed egg masses.

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Discussion

The mapping of the $R_{Mcf\text{-}hou}$ gene involved in resistance against M. chitwoodi (race 1) and M. fallax on potato chromosome 11

In this paper the mapping of the S. hougasii derived $R_{Mcf\text{-}hou}$ gene is described. The straightforward introgression of the gene into the cultivated potato via repeated backcrosses and the simple inheritance of the resistance phenotype clearly show that the resistance to M. chitwoodi race 1 is a monogenic and dominantly inherited trait. Brown et al. (1999) also described the presence of such resistance factors in S. hougasii, and proposed a nomenclature for these R-genes. At this moment the name $R_{Mcf\text{-}hou}$ offers an adequate description, although allelism to the R-gene described by Brown et al. (1999) is not proven. In future it may be required to add more suffixes to the name, such as $R_{Mcf\text{-}hou}11.1$ to indicate also the map position on chromosome 11 and a serial number, if additional genes are localised on this chromosome.

The $R_{Mcf\text{-}hou}$ locus was not only effective to M. chitwoodi population Co, but also against a M. fallax population. This combined resistance to M. fallax and M. chitwoodi has been described before for the resistance gene $R_{mcf\text{-}alc}$ of S. bulbocastanum (Janssen et al. 1997a, Rouppe van der Voort et al. 1999) and $R_{mcf\text{-}den}$ of S. fendleri (Chapter 5). This could indicate that a single gene at this locus can recognise similar or identical avirulence gene products of M. chitwoodi and M. fallax. Alternatively, this locus could contain two closely linked R-genes, which is not uncommon since R-genes are frequently organised in clusters. It is not inconceivable that co-evolution between host and parasite resulted in divergence of the virulence factors which is matched by gene duplication in the R-gene cluster and co-divergence of the duplicated R-genes. When future research will reveal the existence of an individual R-gene for M. fallax, it should be named $R_{Mf\text{-}hou}$.

The S. hougasii derived mapping population RH4X-078 was analysed only with M. chitwoodi population Co, whereas the resistant parent also resisted the populations Ca, Cat and Cba (race 1), and the population Cbd (race 2). Therefore it is tempting to speculate on the presence, segregation and localisation of other resistance genes present in this mapping population. Two arguments plead against this. First, the independent work on three taxonomically distinct potato species did not result in the detection of more than one locus. Second, it did not result in the detection of differential gene interaction between the R_{Mcf} genes and different nematode populations, including race 1 and race 2, and resistance breaking mono-female lines.

The resistance gene $R_{Mcf\text{-}hou}$ was mapped relative to 14 AFLP markers which were obtained using bulked segregant analysis of 394 AFLP primer combinations. In view of theoretical expectations on the efficiency of BSA in tetraploid potato (Li et al. 1998) the rate of 14 out of 394 should be regarded as highly successful, and comparable to their rate of 11 markers out of 205 primer combinations. Elevated DNA sequence dissimilarity between S. hougasii and S. tuberosum could be responsible for this success rate. The R-gene is located at the distal end of the linkage group, and fortunately enough there are flanking markers allowing applications such as marker-assisted selection and map based cloning.

The alignment of the linkage group to chromosome 11, using the strategy of co-migrating AFLP markers, was not successful when using S. tuberosum related reference material. However, when S. hougasii fingerprints were compared with fingerprints from a S. fendleri derived mapping population, the correct linkage group could be assigned. Earlier statements about the utility of a catalogue of co-migrating AFLP fragments (Rouppe van der Voort et al., 1998a) made clear that reference material should represent germplasm with a sufficient degree of fingerprint similarity. Apparently, at this locus, S. fendleri (series LONGIPEDICELLATA Buk.) and S. hougasi (ser. DEMISSA Buk.) are more similar to each other as compared to cultivated potato (ser. TUBEROSA Hawkes). The AFLP marker P+GG/M+CAC_302 that mapped near the resistance gene $R_{Mc1\text{-}hou}$, was also polymorphic in the S. fendleri mapping population were it co-segregated with the resistance gene $R_{Mc1\text{-}fen}$ on chromosome 11 (Chapter 5). An additional CAPS marker M39b confirmed the chromosome number of the linkage group. This marker mapped at a distance of 2 cM to the resistance gene, a distance that is equal to the distance between this marker and the M. chitwoodi resistance gene $R_{Mc1\text{-}blb}$ from S. bulbocastanum (Rouppe van der Voort et al. 1999).

All three R_{Mc1} genes appeared to localise in the same distal region of the long arm of chromosome 11. This region is a well known hotspot of resistance genes, including the white potato cyst nematode resistance gene $R_{0pa\text{-}tar}$ (Wolters et al. 1998), the potato virus Y resistance genes Ry_{sto}, Ry_{adg} and Na_{adg} (Hämäläinen et al. 1997; Brigneti et al. 1997; Hämäläinen et al. 2000), and the wart disease resistance gene Sen1 (Hehl et al. 1999). Such numbers of resistance genes residing in such a small region on the genome, will severely complicate the pyramiding of resistance genes. The selection of valuable recombinants, which carry multiple resistance genes in coupling phase, should be pursued with the assistance of DNA markers.

Comparative analysis of the resistance spectrum of R_{Mc1}-genes

From the potato breeding perspective the value of resistance genes is defined by their ability to avoid yield reduction and to prevent the propagation of pathogen inoculum as well as by the spectrum of isolates they give resistance to. To investigate the spectrum width the $R_{Mc1\text{-}hou}$ gene was tested against six root-knot nematode populations. The gene does not confer broad-spectrum resistance against all populations of M. chitwoodi, because race 1 isolate Cbh and race 2 isolate Ccl could produce egg-masses. The number of populations that are available for M. fallax is too limited to allow generalisations on broad-spectrum resistance of $R_{Mc1\text{-}hou}$ against M. fallax.

When the spectrum of the $R_{Mc1\text{-}hou}$ gene is compared with that of the genes $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}blb}$, it is clear that all genes have a similar effect on the six nematode populations. In view of this result it does not seem advantageous to potato breeders to start pyramiding the $R_{Mc1\text{-}hou}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}blb}$ genes. Further research is needed to find out if combinations of these three genes could have a synergistic effect, and recognise different virulence factors. Alternatively, the cloning of these genes will allow the identification of putative differences between these genes at the level of the DNA sequence. In view of the strong conservation of R-gene sequences

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within R-gene clusters (Bakker 2003) strong homology could be expected, in spite of the large taxonomic distances between the series BULBOCASTANA (Rydb.) Hawkes, LONGIPEDICELLATA Buk., and DEMISSA Buk. from which S. bulbocastanum, S. fendleri and S. hougasii are members.

Comparative analysis of nematode populations

A series of six nematode populations was analysed with susceptible potato and R_{Mc1} genes from three different Solanum species. Difference in virulence between race 1 and race 2 was not found between the races but within. Both within race 1 and race 2 populations were available (Ccl and Cbh) that could produce egg-masses. The population Cbh (also known as CAMc2) has been described as a resistant breaking race 2 population. It was able to multiply on the S. bulbocastanum genotype SB22 (Mojtahedi et al. 1994). We showed that Cbh could overcome the resistance of all three wild species.

Populations Ccl and Cbh seem to have a similar virulence spectrum, although they have been described initially as race 1 and race 2, respectively. Van der Beek et al. (1998b) tested eight Dutch M. chitwoodi populations with the differentials to distinguish race 1 from race 2. They found no indication for race 2 in the Netherlands. Population Ccl has not yet been tested on alfalfa cv. Thor to exclude the possibility that a small fraction of the individuals in population Co belongs to race 2 (Ccl is a mono-female isolate derived from Co, Janssen et al. 1998).

On the basis of the data summarised in Table 3 we have not been able to detect significant interaction between resistant potato genotypes and nematode populations, except for S. bulbocastanum genotypes with a significant different response to Cbd. One possible interpretation could be the presence of a low fraction of resistance breaking individuals in Cbd, and the presence of an additional factor involved in resistance in genotypes 95-B3-03 and 95-B3-13R.

At this moment two virulent populations Cbh and Ccl are available. Population Cbh is a natural population and Ccl is an artificial isolate selected as a mono-female line from egg-masses that are occasionally formed on resistant genotypes upon inoculation with a natural population.

Also in the M. fallax population, occasionally egg-masses are formed on resistant plants, but these egg-masses have not been reared to test the ability of M. fallax to generate virulence breaking mono-female populations. The data from Table 3 show that all avirulent populations Ca, Cat, Cba and Cbd could form egg-masses in low amounts. The same phenomenon was observed for the population Co that was commonly used for the mapping of resistance in S. bulbocastanum (Rouppe van der Voort et al. 1999), S. fendleri (Chapter 5), and S. hougasii (this study).

Durability of the $R_{Mc1\text{-}bcu}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}cbs}$ resistance genes

The occasionally formed egg-masses may have great implications for the durability of the R_{Mc1} genes. Possibly, those egg-masses result from an avirulent nematode that escaped the

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resistance response in the plant. For several plant-pathogen systems incomplete expression of resistance has been described. A more plausible explanation for the occasional production of egg-masses by Ca, Cat and Cba is the presence of a small fraction of virulent individuals, in analogy with the origin of Cbh and Ccl.

Without crop rotation, the fraction of virulent nematodes in a population will increase dramatically, due to the strong selection imposed by resistant cultivars. However, selection imposed by alternative (resistant) hosts, grown in the years without potato, could reduce these effects on population allele frequencies. Cyclical selection can result in trajectories of gene frequencies that fluctuate around an equilibrium point (Leonard 1977).

In addition to forecast the durability of R-genes, the development of mono-female lines is an important tool for both the categorisation of the different virulence factors in M. chitwoodi populations and the identification of R_{Mc1} genes in wild Solanum species. In this study we used the virulent populations Cbh and Ccl to screen potato genotypes. We observed that these two populations were able to circumvent the resistance present in S. fendleri, S. hougasii and S. bulbocastanum derived breeding lines, confirming the results of Janssen et al. (1999).

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Chapter 7

General Discussion

Introduction

The aim of the research described in this thesis is the development and implementation of molecular markers in potato breeding. Although molecular marker development is ongoing for many years, the practical use in Marker Assisted Selection (MAS) in potato breeding is rare. Our study is not just another attempt to map resistance loci but also an attempt to implement MAS in a potato breeding programme. The ultimate goal is the introduction of potato cultivars with resistance to the root-knot nematodes Meloidogyne hapla, M. fallax and M. chitwoodi, selected with molecular markers. In the current potato cultivars resistance to these nematodes is not available. The results described here will contribute to a more rapid development of resistant potato cultivars. The selection in breeding material based on genotype instead of phenotype and selection in seedling stage are ultimate tools for a plant breeder as will be discussed in this chapter. MAS will not replace classical breeding but it will become a tool for the selection at an earlier stage, eventually cheaper and providing new opportunities in resistance breeding by pyramiding genes. The strategy followed in marker development, the durability of the resistance and the consequences of the implementation of molecular markers in potato breeding, will be discussed.

Potato breeding

The most important characters in potato breeding are a high yield combined with good quality. The quality depends on the different breeding objectives like French fries, chips, fresh consumption and starch potatoes. Within these objectives the requirements of quality can also depend on local preferences that differ per country. Besides the quality and other agronomical traits, resistances to diverse pathogens and pests are important. Selection for resistances and agronomical traits are almost always made based on the phenotype of the clones. The potato clones are, therefore, tested in the field for the agronomical traits and in bioassays in field and laboratory for the different diseases and pests. In some area's resistance to certain pests are a prerequisite like wart disease and potato cyst nematode resistance in starch potato cultivars grown in the north east of The Netherlands. Therefore the first bioassays are already performed after two years of selection. Because the amount of produced seed potatoes is limited, the bioassays will then be performed in just one replication. Especially the two mentioned traits are very sensitive to the environment, which implies that more replications should be made. These resistance tests will therefore be repeated the next years in the breeding programme with every year in succession more replications. Because the large amount of tests the breeding of a new potato cultivar is a slow process. It takes approximately 9 to 12 years from making the cross to the admission for the list of varieties. New techniques to speed up the breeding process are available but not yet fully implemented into the breeding process. One of these techniques is marker assisted selection (MAS). The molecular markers which are diagnostic for certain traits are identified by genetic analysis of segregating populations. These molecular markers can eventually lead to the cloning of genes and subsequently the genetic modification of a variety. Genetically modified varieties with an enhanced resistance to diseases and pests should be an acceptable solution to reduce the chemical input in agricultural production. Regrettably due to the opposition of certain environmental groups the GMO approach in potato has been limited in The Netherlands to one potato with a modified starch composition to be used for industrial

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purposes. Until the time genetic modification of plants will be accepted, the potato breeders have to incorporate resistance in potato cultivars by means of traditional breeding methods. In order to speed up the resistance breeding, they can use MAS.

Resistance breeding in potato

The first step in resistance breeding is the identification of genetic variation within potato cultivars and within its wild relatives. Sufficient genetic diversity is available in the Solanum species including resistances to diseases and pests that can be used for the introgression of resistance into potato cultivars. Many of these wild potato species have a diploid chromosome level, in contrast to the common potato cultivars that have a tetraploid level. The use of a diploid potato breeding programme allows direct gene transfer from these wild diploid Solanum species to S. tuberosum. An additional advantage of breeding at the diploid level is the more efficient backcrossing process, necessary after such an intraspecific cross. At the diploid level the offspring will also display a wider spectrum of phenotypic variation, which implicates a smaller progeny size and a lower number of backcrosses needed. Besides the more efficient breeding, the use of diploid potatoes simplifies the genetic research compared with tetraploid potato, specifically when multiple genes are involved in the character studied. In the research described in this thesis polyploid as well as diploid wild species are used.
We followed the strategy of combining introgression breeding and genetic research. The genetic research should reveal the loci of the resistance genes. After developing closely linked markers, MAS can be applied to speed up the breeding process. Resistant hybrids are used for further crossing with potato clones and selection on agronomical traits will be done phenotypically on the resistant progeny of these crossings. Although interspecific backcrosses can be complex due to crossings barriers, the application of this strategy will lead to the introgression of the resistance which is a goal on its own. Moreover, when polyploid wild species are used, interspecific backcrosses will lead to a simplex/nulliplex condition of the target gene, as well as for the markers, as required for BSA. In Chapter 5 two strategies are compared by generating both an intraspecific and interspecific mapping population for the detection of resistance loci. Although we have a limited amount of data, the results support the commonly observed reduced level of recombination between the introgression segment and the recipient genome of interspecific introgression material (De Vicente & Tanksley 1991; Ganal & Tanksley 1996). In the same chapter we observed an elevated level of polymorphisms in the interspecific mapping population. This can be due to the fact that S. fendleri is a self-fertilising species, so in an intraspecific mapping population an elevated level of homozygosity is expected. Additionally the dissimilarity between the wild species introgression segments and the recurrent potato parents in the interspecific mapping population may have caused an elevated level of polymorphic markers.

Root-knot nematode

After P. infestans the second important pest in Western Europe is the potato cyst nematode Globodera rostochiensis and G. pallida. Potato breeders have been very successful in breeding for resistance to G. rostochiensis. Most of the Dutch potato starch cultivars and a few consumption potato cultivars are resistant to G. pallida. When farmers choose resistant

cultivars they should be able to control the potato cyst nematode problem. This choice should be based on the identity of the resistance gene and the avirulence of the nematode population. A good variety management will eventually lead to a more cost efficient and environmentally safe way of producing potatoes. The use of resistant cultivars already led to a substantial reduction in chemical use in the intensive potato cultivation. This has however also led to an increasing problem with other nematodes like Pratylenchus penetrans, P. crenatus, Trichodorus spp. and the root-knot nematodes Meloidogyne chitwoodi, M. fallax and M. hapla. The most abundant of these nematodes are the Pratylenchus and Trichodorus species. The root-knot nematodes are however also an increasing problem, especially in the southern part of The Netherlands where almost 25% of the investigated soil samples of the fields with assumed nematode problems, contained among others M. chitwoodi nematodes (pers. com. J. Peltjes, HLB). The sandy soils and the intensive cultivation of potatoes also makes the circumstances in the north east of The Netherlands favourable for the increase and spread of M. chitwoodi and M. fallax. In contrast to nematodes like Pratylenchus spp. and Trichodorus spp., the root-knot nematode can be transferred with the seed potatoes. The risk of spreading the root-knot nematodes by seed potatoes is especially large when the production of seed potatoes is based on the regulation of farmer saved seed. This method of seed production has a minimal phytosanitary control by the government and is therefore an important factor in the spread and increase of the root-knot nematodes. In addition most of the potatoes grown in the starch producing area are late maturing varieties, which offer the nematodes the possibility of more generations and therefore a larger increase of the population. Under favourable conditions M. chitwoodi can have up to 4 generations per growing season (Pinkerton et al. 1991). The problems with M. hapla in the potato cultivation seem less critical as compared with M. chitwoodi and M. fallax. The occurence in The Netherlands of M. hapla is less frequent than the other two root-knot nematodes. In contrast to the swelling or galls and discoloration of the flesh caused by M. chitwoodi and M. fallax, the damage caused by M. hapla is limited to yield loss. This reduction in yield is however not always recognized by the farmer as a result of M. hapla infestation. In areas where potato is part of a crop rotation with crops like carrot, pea, black salsify, onion or chicory, M. hapla may cause more problems, due to damage in the follow up crop after the increase of the nematode population during the growth of potato.

Strategies for controlling the root-knot nematode

The control of the root-knot nematodes by means of chemical fumigation is an effective and economically feasible method but from an environment point of view undesirable. An alternative method of control is the use of resistant green manure crops, like winter radish. It is known that there are differences in multiplication among varieties of this crop but more research is required to assess the economical feasibility of this method. Another possibility to reduce the damage of root-knot nematodes is a wider crop rotation. Crop rotation however might be only effective to control M. hapla which does not multiply on monocotyledons. Cultivation of monocotyledons is most likely the reason why M. hapla is not frequently found in the north east of The Netherlands. The broad host range of M. chitwoodi and M. fallax makes crop rotation more complicated as they multiply on most of the crops used in common rotation schemes like sugar beet, wheat and barley. Looking at the larger problem with M. chitwoodi and M. fallax, the

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