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Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato

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Summary

Comparative genomics provides a tool to utilize the exponentially increasing sequence information from model plants to clone agronomically important genes from less studied crop species. Plant disease resistance (*R*) loci frequently lack synteny between related species of cereals and crucifers but appear to be positionally well conserved in the Solanaceae. In this report, we adopted a local RGA approach using genomic information from the model Solanaceous plant tomato to isolate *R3a*, a potato gene that confers race-specific resistance to the late blight pathogen *Phytophthora infestans*. *R3a* is a member of the *R3* complex locus on chromosome 11. Comparative analyses of the *R3* complex locus with the corresponding *I2* complex locus in tomato suggest that this is an ancient locus involved in plant innate immunity against oomycete and fungal pathogens. However, the *R3* complex locus has evolved after divergence from tomato and the locus has experienced a significant expansion in potato without disruption of the flanking colinearity. This expansion has resulted in an increase in the number of *R* genes and in functional diversification, which has probably been driven by the co-evolutionary history between *P. infestans* and its host potato. Constitutive expression was observed for the *R3a* gene, as well as some of its paralogues whose functions remain unknown.

Keywords: *R3a*, comparative genomics, potato, tomato, disease resistance, *Phytophthora infestans*.

Introduction

Comparative genomics investigates the similarity and differences in structure and function of genomes across taxa. Full genome sequences of Arabidopsis (Arabidopsis Genome Initiative, 2000) and rice (Goff *et al.*, 2002; Yu *et al.*, 2002) and several other ongoing sequencing projects will offer unprecedented resources to study the evolution of sequence and function of orthologous genes and to understand diversification and adaptation. A fundamental and practical question in comparative genomics is whether this vast amount of sequence information from model plant species will facilitate the cloning of genes with agronomic importance from crop species with larger genomes. Resistance to plant pathogens, often defined by single dominant disease resistance (*R*) genes (Dangl and Jones, 2001), is an important crop trait that could benefit from the sequencing of model species. This benefit, however, will largely depend on the given plant family. Extensive loss of colinearity has been reported within crucifers and grasses (Gale and Devos, 1998; Paterson *et al.*, 2000). Comparative analysis based on DNA sequences has revealed that disease resistance (*R*) loci may be evolving faster than the rest of the crucifer and grass genomes (Gale and Devos, 1998; Leister *et al.*, 1998; Paterson *et al.*, 2000). In contrast, the remarkable conservation of gene order (Bonierbale *et al.*, 1988) makes the Solanaceae, a botanical family that includes many important crops such as tomato and potato, an attractive subject for comparative genomics. Low-resolution comparative mapping indicated that *R* loci may be positionally conserved within Solanaceae (Grube *et al.*, 2000; Leister

et al., 1996; Pan *et al.*, 2000). To date, this knowledge has never led to the isolation of a new *R* gene with known function.

The distal end of the long arm of chromosome 11 of tomato is a particularly interesting genomic region to investigate the evolution of *R* loci in a comparative genomics perspective. First, the overall structural colinearity is excellent between tomato and potato (Tanksley *et al.*, 1992) and relatively good between tomato/potato and pepper (Livingstone *et al.*, 1999). Secondly, this genomic region is a hotspot for *R* genes, harboring major genes encoding resistance to the fungi *Fusarium oxysporum* (*I2*, Ori *et al.*, 1997; Simons *et al.*, 1998) and *Stemphylium* spp. (*Sm*, Behare *et al.*, 1991), and to yellow leaf curl virus (Hanson *et al.*, 2000) in tomato, to the oomycete *Phytophthora infestans* (*R3, R6,* and *R7,* El Kharbotly *et al.*, 1994, 1996) in potato, and to tobacco mosaic virus (*L*, Lefebvre *et al.*, 1995) in pepper. The region also contains several quantitative trait loci conferring resistance to the cyst nematode *Globodera rostochiensis* (*Gro1.3*, Kreike *et al.*, 1993), to *P. infestans* (*phyt7*, Oberhagemann *et al.*, 1999) in potato, and to cucumber mosaic virus (*cmv4*, Grube *et al.*, 2000) and to *P. capsici* (*phyt 3*, Lefebvre and Palloix, 1996) in pepper. Last, the molecularly well characterized *I2* complex locus in tomato (Ori *et al.*, 1997; Simons *et al.*, 1998) provides an excellent template for a comparative study. The complex locus consists of two clusters, *SL8D* and *SL8E*. The *SL8D* cluster contains seven coiled-coil nucleotide binding site and leucine-rich repeat (CC-NBS-LRR) type *R* gene sequences, including the *I2* gene conferring complete resistance to race 2 of *F. oxysporum* f. sp. *lycopersici* (Simons *et al.*, 1998) and the *I2C-1* (Ori *et al.*, 1997) and *I2C-5* (Sela-Buurlage *et al.*, 2001) genes conferring partial resistance to the same pathogen.

Potato is the most important non-cereal crop plant. One of the major constraints to potato production in the world is the late blight disease caused by the oomycete *P. infestans* (Fry and Goodwin, 1997). There is considerable interest in identifying late blight *R* genes and in understanding their evolution and mode of action (Ballvora *et al.*, 2002; Song *et al.*, 2003; van der Vossen *et al.*, 2003). In a previous study (Huang *et al.*, 2004), we discovered that the *R3* complex locus at chromosome 11 of potato comprises two functionally distinct late blight *R* genes, *R3a* and *R3b*. Here, we use genomic information from the model Solanaceous plant tomato to clone *R3a*, a gene that confers race-specific resistance to *P. infestans*.

Results

The potato R3 *and tomato* I2 *regions are highly colinear*

We previously showed that the genomic regions harboring the *R3* late blight resistance locus in potato and the *I2* *Fusarium* wilt resistance locus in tomato are colinear (Huang *et al.*, 2004). To determine the extent of colinearity between these regions, we mapped seven tomato markers on the high-resolution genetic map of the *R3* complex locus (Figure 1). All seven markers retained their order in tomato and potato. We identified a cluster of *I2* gene analogues (*I2*GAs) in potato that was mapped 0.1 cM centromeric to the TG105 marker. This potato *I2*GA cluster positionally corresponds to the *SL8D* cluster of the *I2* complex locus in tomato and was therefore named the *St-I2* cluster. The perfect micro-colinearity within the TG105-cLET24J2A interval indicates that the *R3a* cluster in potato is syntenic to the *SL8E* cluster in tomato. Despite the fact that the counterpart of the *R3b* cluster was not detected in tomato, the orthologous relationships of *SL8D* versus *St-I2* and *SL8E* versus *R3a* point to an ancient *R* locus prior to the tomato–potato divergence.

R3a *candidates were identified using a local resistance gene analogue (RGA) approach*

Although the potato *R3a*-tomato *SL8E* synteny was established (Figure 1), we could not directly use it for *R3a* cloning as there was no sequence available from the *SL8E* cluster.

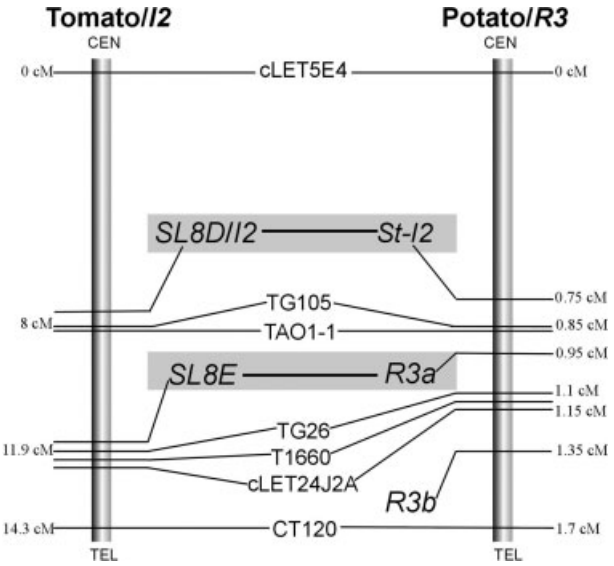

Figure 1. Comparative genetic maps of the *I2* complex locus in tomato and the *R3* complex locus in potato. The left and right solid bars represent the tomato and potato chromosomes, respectively. Orientation is indicated by CEN (centromere) and TEL (telomere). Mapped markers are connected by black lines. The syntenic relationships of *R* gene clusters are highlighted using gray rectangles. On the tomato map, the positions of TG105, TG26, and CT120 are according to Tanksley *et al.* (1992), cLET5E4, T1660, and cLET24J2A according to the Solanaceae Genomics Network (http://www.sgn.cornell.edu), and *SL8D* (containing the *I2* gene), TAO1-1, and *SL8E* according to Ori *et al.* (1997). On the potato map, the positions of cLET5E4, TG105, *R3a*, TG26, *R3b*, and CT120 were determined by a previous work (Huang *et al.*, 2004), TAO1-1, T1660, and cLET24J2A by aligning the marker sequences to the sequences of BACs mapped on the high-resolution map of *R3* (data not shown). The position of *St-I2* was determined as described in the text.

However, the *SL8E* cluster was defined by cross-hybridization with the LRR part of the *I2C-1* gene from the *SL8D* cluster (Ori *et al.*, 1997). Using this information, a local RGA approach was applied. Instead of targeting conserved motifs within the NBS applied by global RGA approaches (Aarts *et al.*, 1998; Kanazin *et al.*, 1996; Leister *et al.*, 1996; van der Linden *et al.*, 2004; Yu *et al.*, 1996), the conserved sequences within the LRR of the *SL8D* cluster were used to design the P_{SL8D} primers (Table 1C). A BLAST search (Altschul *et al.*, 1990) for short, nearly exact matches proved the specificity of the P_{SL8D} primers, which hit no sequence other than the *SL8D* cluster with a reasonable low *E*-value. On DNA from the parental clones SH83-92-488 (SH) and RH89-039-16 (RH), the primers amplified a major smearing band of approximately 1 kb (data not shown), indicating that many copies of *I2*GAs with similar sizes were amplified. The size fitted the prediction from the sequences of the *SL8D* cluster (884–1049 bp). The above findings confirmed the suitability of the P_{SL8D} primers for identifying *I2*GAs in potato.

To identify *I2*GAs physically close to *R3a*, an association analysis on bacterial artificial chromosome (BAC) pools was conducted. Similar to other physical mapping methods like radiation hybrid mapping (Cox *et al.*, 1990) or HAPPY mapping (Dear and Cook, 1993), our method is based on the frequency that a given marker and *I2*GAs co-appear in BAC pools to estimate physical distance between them. The mapping panel is represented by BAC pools that contain 384 BACs, equivalent to 0.05 genome.

To determine whether any *R3a* marker (Figure 2a) was physically close to an *I2*GA, we screened 255 BAC pools with

Table 1 Molecular markers and primers used, their sequence, annealing temperature (T_m), and restriction enzyme used to generate polymorphism

	Marker	Primer (5′–3′)	T_m (°C)	Restriction enzyme
A	cLET5E4	CCA GGC ATG CTC AAT TTG GAG T TTC CCT GTT TGG ACT ACT TGT GGA	55	*Hha*I
	CT120	CGA GGG GGC GAA GGA TT CCA TGA GAT AAA CGA GGA ACC AGT	52	*Tsp*509I
B	241S	GGC ACT GAT AAG TTT TGG TTT TG GTG GTT TAT GAA TTG AAC TCA TGC	55	a.s.[c]
	194T	AAG CTT GAA TTC GTG GAC GG CTT TAT ACC AAC AGG TTG CTC	55	a.s.
	4T	AAG CTT TCA AAC CAA AAT GC AAA ATG ACT TTA CGT GGT CT	52	a.s.
	GP250	ACC AGT AGG ACC ACC ACC AAC AAT GAT CGT GAC GGC TCT ACT CTT TTA TGA	60–52[b]	*Vsp*I
	GP250-2	CTG GTA ATA GTA GTA ATG ATT CTT CGT C GAT CCT GAC TGC TCT ACT CTC TTA CGA	60–52[b]	*Vsp*I
C	P_{SL8D}	AAT TGG AGA GTT CCC TAC ACT TGA G AGG GAG GAG GGC AGT GCT GAT TC	50	*Alu*I
D	3RACE4	CGA AAG GAG TGG CAT TTA CAG AGA CGA	68–60[b]	
	3RACE5	CTT CCT CTA TTC AAA GGC TTA CCA TAG TG	68–60	
	3RACE6	CTC ACC TCT CTT CAA TAT CTA TTT ATT AGG	68–60	
	5RACE0	CAT TGT AAA CCG CTT TAG CAA GTG TTG TC	68–60	
	5RACE4	CAT TCC AAC AAT AGG AAC TAC AGT CCG C	68–60	
	5RACE5	CAT TCT GCC TTC CAA AGA TAT CAG AG	68–60	
	5RACE6	CAT CAC TTA GCA CAA TCT GAA GAC CG	68–60	
E	RT-SH23-1	GCT TCC GAT ATG TAT TGA TCT CAC G TGT GGC AAT CCT CTA CAA ACA ATG T	68–55[b]	
	RT-R3a	ATC GTT GTC ATG CTA TGA GAT TGT T CTT CAA GGT AGT GGG CAG TAT GCT T	68–55	
	RT-SH23-3	CGA CAT GTA ATT ATT TCA TGC CTC C AGA GGA ATT TCA AAC AAG GGA GTT C	68–55	
	RT-SH194-2	TGG ATT GAA AAG TTG CCT GAT TCT G CAA GGT AGT GGG CAG TAT GCT AAA T	68–55	
	Actin[a]	CAG CAA CTG GGA TGA TAT GG ATT TCG CTT TCA GCA GTG GT	68	

A, flanking markers; B, physical mapping; C, RGA marker; D, RACE; E, RT-PCR.
[a]Primers kindly provided by Chengwei Li of the Laboratory of Plant Breeding, Wageningen University.
[b]Touchdown PCR, −1°C/cycle.
[c]Allele-specific.

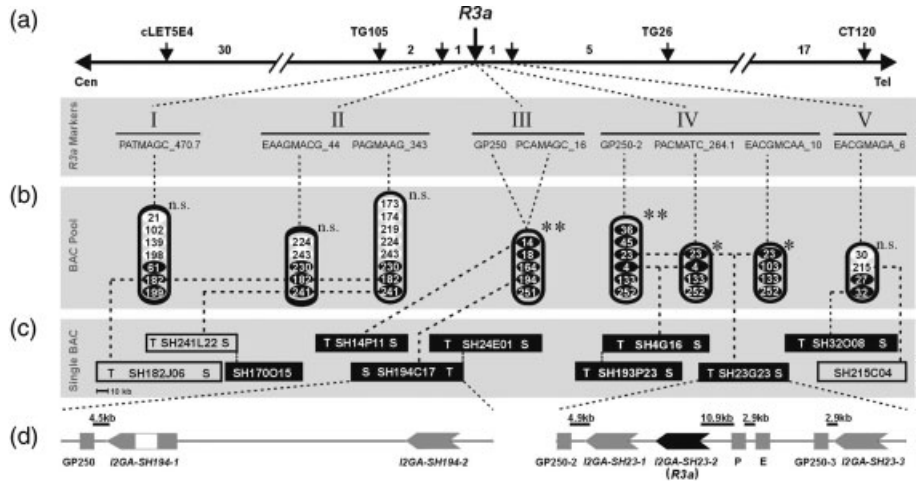

Figure 2. Genetic and physical mapping of *R3a*.

(a) Genetic map of *R3a*. Vertical arrows indicate the relative positions of markers linked to resistance. Numbers between the markers above the horizontal line indicate the number of recombinants identified in the 1748 SH × RH progeny used in a previous study (Huang *et al*., 2004) and the additional 1200 progeny used in this study. The *R3a* markers are divided into five groups (I–V) (described in the text). GP250-2 was found in BAC pool screening, which is a copy of GP250 and also cosegregates with *R3a*.

(b) Physical association of markers and *I2*GAs. Oval beads within round-end rectangles represent set of BAC pools identified by a given marker in groups I–V. BAC pools with *I2*GA(s) are represented by a black background. The extent of association (n.s., not significant, $*P < 0.05$, $**P < 0.01$) between a given *R3a* marker and *I2*GAs on BAC pools are indicated on the upright side of each round-end rectangle.

(c) BAC Contigs at the *R3a* region. Rectangles present BACs and those carrying *I2*GA(s) are highlighted using black background. T (T7) and S (Sp6) indicate the orientation of the BACs. BACs SH182J06 and SH241L22 were identified by group I/II markers, SH14P11 and SH194C17 by group III markers, SH4G16 and SH23G23 by group IV markers, and SH32O08 and SH215C04 by group V marker. SH170O15, SH24E01, and SH193P23 were identified using BAC end markers 241S, 194T, and 4T (Table 1B), respectively.

(d) Location and orientation of I2GAs in SH194C17 and SH23G23 are shown. Single continuous ORFs were identified in *I2GA-SH194-2*, *-SH23-1*, *-2*, and *-3*. I2GA-SH194-1 is inserted by a retroelement (indicated by the white block). The positions of the markers GP250 and GP250-2 were identified by alignment. GP250-3 was found as another copy of GP250. The positions of the group IV AFLP markers EACGMCAA_10 (E), and PACMATC_264.1 (P) were defined *in silico* by identifying their restriction sites, selective nucleotides, and mobilities. The distance between markers and I2GAs is indicated above the annotated BACs.

the nine *R3a* markers and the P_{SL8D} primers. A total of 30 BAC pools were determined to be positive for one or more of the *R3a* markers, placing these markers in five groups on the basis of recombination events and co-occurrence in BAC pools (Figure 2a,b). The P_{SL8D} primers identified 92 *I2*GA-containing BAC pools, 19 of which coincided with those positive for the *R3a* markers. Chi-square tests showed that *I2*GAs were significantly associated with the markers in groups III and IV that cosegregated with *R3a* (Figure 2b). This suggested a close physical relationship between *R3a* and specific *I2*GAs, which therefore were regarded as *R3a* candidates.

From the BAC pools identified by the *R3a* markers and by chromosome walking, we isolated 11 BAC clones that span at least 700 kb (Figure 2c). Subsequently, BAC clone SH194C17 and SH23G23 were chosen for sequencing, as they were respectively identified by group III and IV markers (Figure 2c) and Southern hybridization (data not shown) indicated they contained five *I2*GAs. Annotation of the BAC sequences indeed predicted five *I2*GAs that were designated *I2GA-SH194-1*, *-2*, *I2GA-SH23-1*, *-2*, and *-3* (Figure 2d). Single continuous open reading frames (ORF) were present in *I2GA-SH194-2* and the three *I2*GAs in BAC SH23G23. *I2GA-SH194-1* is a pseudogene due to insertion of a Ty1/Copia-type retroelement. In the BAC sequences, the GP250 marker and the amplified fragment length polymorphism (AFLP) markers could also be recognized. The physical proximity between the *I2*GAs and the group III/IV markers (Figure 2d) validated the predicted association at the level of BAC pools (Figure 2b). The four *I2*GAs with full ORFs were considered as *R3a* candidates.

R3a *is an* I2*GA*

The genes *I2GA-SH194-1* and *-SH23-1*, *-2*, *-3* together with 2–3 kb up- and down-stream sequences were introduced into the susceptible clone 1029-31 and cv. Desiree via *Agrobacterium*-mediated transformation. At least 10 *in vitro* plantlets of each primary transformant were tested for resistance to each of the three *P. infestans* isolates 89148-9, IPO-0, and H30P04 in duplicate experiments. Cv. Desiree, 1029-31, RH, SH, and SW8540-025 (*R3a* recombinant) were included as controls. Only a 10.4-kb subclone containing *I2GA-SH23-2* was able to specifically complement the susceptible phenotype. All 15 primary transformants of this subclone exhibited *R3a*-specific resistance, that is, were resistant to isolate 89148-9 and IPO-0 and susceptible to H30P04 (Table 2 and Figure 3). The results were confirmed using a detached-leaf assay in duplicate tests. As the subclone contained only one ORF, we designated *I2GA-SH23-2* as *R3a*.

Table 2 Disease test of primary transformants of *R3a* and its paralogues. Numbers of plants showing resistance to the three *Phytophthora infestans* isolate IPO-0, H30P04, and 89148-09 are indicated

Construct	Clone	No. plants tested[a]	No. plants showing resistance to *P. infestans* isolate[b]		
			89148-09	IPO-0	H30P04
*I2*GA-SH23-1	1029-31	12	0	0	0
	Desiree	6	0	0	0
*I2*GA-SH23-2 (*R3a*)	1029-31	8	8	8	0
	Desiree	7	7	7	0
*I2*GA-SH23-3	1029-31	13	0	0	0
	Desiree	5	0	0	0
*I2*GA-SH194-2	1029-31	23	0	0	0
	Desiree	8	0	0	0
Control	1029-31	10	0	0	0
	Desiree	10	0	0	0
	RH	10	0	0	0
	SW8540-025	10	10	10	0
	SH	10	10	10	10

[a]For constructs – number of independent transformants; for control – number of *in vitro* plantlets of the same genotype inoculated with each isolate.
[b]89148-09 and H30P04 kindly provided by Dr F. Govers of Laboratory of Phytopathology, Wageningen University and IPO-0 by Dr W. Flier of Plant Research International, Wageningen, The Netherlands.

The transcript structure of the *R3a* gene was determined by comparing the genomic sequence with cDNA fragments generated by random amplification of cDNA ends (RACE). The *R3a* transcript is 4176 nt long and encodes a predicted polypeptide of 1282 amino acids (a.a.) with a relative molecular mass of 145.9 kDa. The *R3a* gene and the *I2* gene (Simons *et al.*, 1998) have a similar intron–exon pattern and both have no intron in the coding region, but the intron close to the stop codon is much longer in *R3a* than in *I2* (Figure 4a).

The *R3a* gene encodes a putative CC-NBS-LRR protein and shares 88% DNA identity and 83% a.a. similarity to *I2* (Figure 4b). Considerable dissimilarity occurs at the CC domain, including a seven-a.a. indel. R3a and I2 proteins are quite conserved in the NBS domain (86% a.a. identity), especially at the motifs that define the domain (94% a.a. identity). The proteins mainly diverge at the LRR domain where two major differences were observed. First, although both proteins carry 29 LRR units, the R3a protein lacks a complete LRR unit of 28 a.a. at LRR 14/15 and contains an extra copy of a unique LRR unit of 23 a.a. at the LRR 26/27 (Figure 4b). Secondly, 45% (63/140) of the putative solvent-exposed residues (x in the frame xxLxLxx) differ between the I2 and R3a proteins.

The R3a and I2 proteins are more related to each other than to other known R proteins, as indicated by the zero *E*-value using BLASTP (Altschul *et al.*, 1990). Thus the *I2* and *R3a* genes belong to the same *R* gene family. Outside of this family, the most related known *R* gene is *Rpg1-b* ($E = e^{-176}$, 35% a.a. identity) from soybean mediating recognition of the Type III effector protein AvrB from *Pseudomonas syringae* (Ashfield *et al.*, 2004). The R3a protein bears only limited similarity (15 and 30% a.a. identity) to the other two known late blight R proteins, R1 (Ballvora *et al.*, 2002) and RB/Rpi-blb1 (Song *et al.*, 2003; van der Vossen *et al.*, 2003), respectively.

Dynamic evolution occurred in the R3a *cluster*

Despite the excellent overall colinearity in the *I2* and *R3* genomic regions (Figure 1), the *R3* region is physically larger in potato. In tomato the TG105-TG26 interval spans about 500 kb and contains one or few *I2*GAs, as demonstrated by long-range physical mapping (Simons *et al.*, 1998) and Southern analysis (Ori *et al.*, 1997). In contrast, the TG105-TG26 interval in SH spans more than 1 MB and contains at least nine *I2*GAs. The three contigs (11 BACs in total) in the *R3a* region (Figure 2c) span at least 700 kb. In addition, gaps between the depicted contigs and those between the depicted contigs and contigs harboring the markers TG105 and TG26 are predicted to be larger than 100 kb, the average

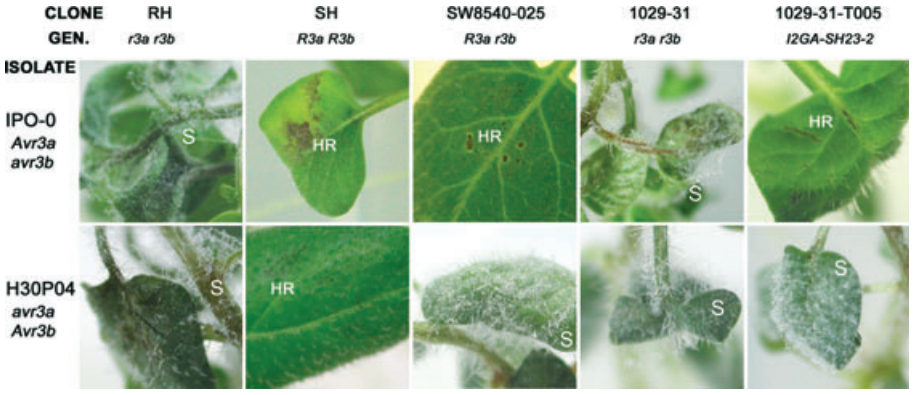

Figure 3. *In vitro* inoculation of the primary transformants of *R3a*. Massive sporulation (S) and localized hypersensitive reactions (HR) are observed on compatible and incompatible interactions, respectively. RH and 1029-31 are susceptible to both isolates. SH is resistant to both isolates. SW8540-025 (an SH × RH progeny carrying only *R3a*) and 1029-31-T005 (a 1029-31 transformant with *I2GA-SH23-2*) are resistant to IPO-0 (with *Avr3a*) and susceptible to H30P04 (without *Avr3a*).

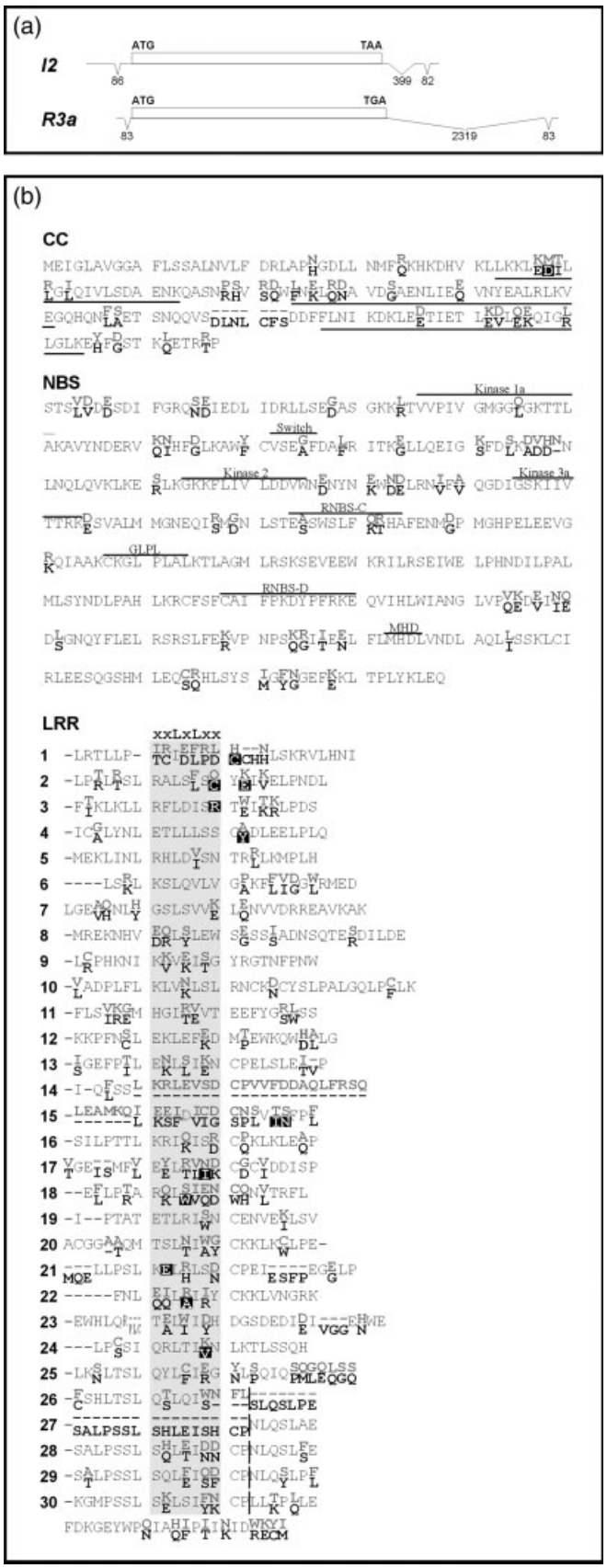

Figure 4. Comparison between *R3a* and *I2*.
(a) Schematic diagram of the transcript structures of *I2* and *R3a*. Horizontal lines indicate exons. Open rectangles represent coding sequences. Lines angled downwards indicate the position of introns, whose sizes are indicated below.
(b) Primary structure and alignment of the I2 and R3a resistance proteins. Amino acid residues of I2 and R3a that are identical are shown in normal script. I2- and R3a-specific residues are shown in bold at top and bottom lines, respectively. Dashes indicate deletion. The predicted coiled coils (Lupas *et al.*, 1991) in the CC domains are underlined. The conserved motifs of the NBS domain are overlined. The 30 LRR units in the alignment are numbered. The β-sheet (consensus xxLxLxx) of each LRR is highlighted with a gray background. The R3a residues under significant diversifying selection are highlighted with a black background. The calculation is based on the alignment of *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2* using PAML (Yang *et al.*, 2000). Between vertical lines are the unique LRR units (consensus NLQSLAESALPSSLSHLEIDDCP) that display distinct copy numbers between *I2*, *R3a*, and their paralogues.

insert size of the SH BAC library. In addition to the five *I2*GAs in BAC clone SH194C17 and SH23G23, we identified a new *I2*GA in each of the four BACs (SH170O15, SH24E01, SH193P23, and SH32O08, Figure 2c) by Southern blotting (data not shown). Altogether, these data indicate that the *R3* complex locus in SH has expanded significantly compared to that of tomato (cv. Motelle and Mogeor, Ori *et al.*, 1997; breeding line E22, Simons *et al.*, 1998), resulting in an increase in the number of *R* gene sequences. It remains unknown whether such variation in size and *R* gene copy number reflects *de facto* tomato and potato genomes or whether such variation also exists within each species.

Diversifying selection has been detected in many *R* gene complex loci (Hulbert *et al.*, 2001). Sites under diversifying selection were investigated using the program PAML (Yang, 1997; Yang *et al.*, 2000). Models M7 and M8 in the program 'codeml' of PAML were run for the four *I2*GAs (*R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*) at the *R3a* cluster in potato. Model M7 is a special case of model M8 and assumes no selection, whereas model M8 allows for positively selected sites (Yang *et al.*, 2000). Diversifying selection can be confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang *et al.*, 2000). Comparison of the results from model 7 and model 8 showed that the I2GAs at the *R3a* cluster have undergone diversifying selection ($\chi^2 = 92.8$, df $= 2$; $P < 0.001$). Selection at each site of the *R3a* homologues was calculated using model M8. Thirteen sites were found to be under significant diversifying selection (Figure 4b and Figure S1). Twelve of them are in the LRR domain and seven are putative solvent-exposed residues. This observation is compatible with the idea that the LRR region of an R protein mainly defines recognition specificities (Parniske *et al.*, 1997; Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2001).

Putative sequence exchange between paralogues was previously observed at several *R* gene complex loci (Noel *et al.*, 1999; Parniske *et al.*, 1997) and is likely a mechanism for creating new specificities (reviewed in Hulbert *et al.*, 2001). Sequence relationships within the *R3a* cluster were analyzed by determining informative polymorphic sites (IPS, Parniske *et al.*, 1997). In total, 129 IPS were detected (Figure S2). The *R3a* and *I2GA-SH23-1* genes have 94% DNA identity and they share 91 IPS, suggesting that they are derived from the same ancestral gene. Interestingly, in the central part of the alignment, the *R3a* cluster exhibits a complex patchwork. The *R3a* and *I2GA-SH194-2* genes share sequence affiliations at three patches, comprising 21 IPS, which result in 10 non-synonymous substitutions between *R3a* and *I2GA-SH23-1*. An almost continuous sequence affiliation (nine IPS) between the *R3a* and *I2GA-SH23-2* genes was also found, comprising three non-synonymous substitutions. It remains unclear whether the putative sequence exchanges between the paralogues have lead to new resistance specificities in the *R3a* cluster.

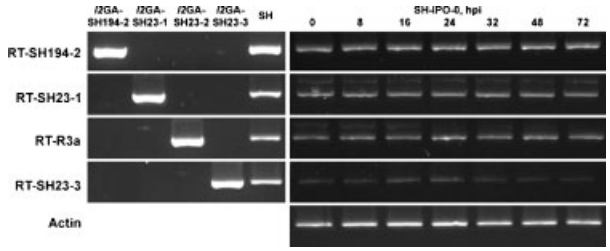

Figure 5. RT-PCR of the *R3a* cluster. Gene-specific primers used are listed on the left. Subclones with individual *I2*GAs (*I2GA-SH194-2*, *-SH23-1*, *-2*, *-3*) and genomic DNA of SH are used as control for the specificities of the primers. mRNA isolated from the detached leaves of SH inoculated with *Phytophthora infestans* isolate IPO-0 were used to study the expression pattern in a time course from 0 to 72 hpi. Actin primers were used to monitor mRNA quantity.

R3a *is constitutively expressed*

To examine the expression patterns of the genes *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*, gene-specific semiquantitative RT-PCR was performed on mRNA isolated with from *P. infestans* challenged (isolate IPO-0 and 90128) or mock-inoculated leaves of SH at 0, 8, 16, 24, 32, 48, and 72 h post-inoculation. All the four *I2*GAs are constitutively expressed in all treatments, but the expression level of *I2GA-SH23-3* is significantly lower than the other paralogues (Figure 5).

Discussion

In this study, we used genomic information from tomato to isolate the potato late blight resistance gene *R3a* from an ancient locus involved in plant innate immunity in the Solanaceae. Despite technological advances in genomics in recent years, positional cloning of *R* genes from large-genome crop species is still far from a routine procedure mainly due to low recombination frequencies and the high repetitive nature that are characteristic of most complex *R* loci (Hulbert *et al.*, 2001). In potato, the heterozygous genome is an additional complicating factor (Kanyuka *et al.*, 1999). To partially circumvent these obstacles, our local RGA approach analyzes genetic markers on BAC pools or superpools, offering enough template complexity in PCR to allow most markers to be allelic- and locus-specific when compared with the hybridization method on single BAC filters. Although the RGA-specific primers (P_{SL8D}) were designed for the exclusive identification of the *I2* gene family, they were also designed to be allele- and locus-insensitive in order to include all candidate RGAs at the target region. Through association on BAC pools, we identified a subset of RGAs that were in coupling phase with and physically close to genetic markers linked to *R3a* and thus 'jump' rather than 'walk' into BACs carrying such RGAs. The success of the method depends on the marker saturation level at the target region and the capacity of the RGA-specific primers to

amplify all the candidate RGAs at the target region. In this case, we benefited from the marker saturation provided by the ultra-high dense (UHD) AFLP potato map (Isidore *et al.*, 2003), as the same mapping population (SH × RH) was used to generate the UHD map and for the initial mapping of *R3a* (Huang *et al.*, 2004). In general, a bulked segregant analysis (Michelmore *et al.*, 1991) in combination with AFLP marker technology will generate adequate marker saturation of the *R* gene regions as the 'wild' *R* haplotype often differs a lot from the 'domestic' *r* haplotype, which is also the reason that they hardly recombine. The synteny at the target region between tomato and potato was beneficial to the RGA approach, despite extensive expansion of the *R3* complex locus compared to the syntenic *I2* complex locus.

Most of the molecularly characterized *R* genes belong to families of tightly linked genes and at many such complex loci, most paralogues appear to encode proteins similar to the functional *R* genes (Hulbert *et al.*, 2001). We demonstrated that all four paralogues of the *R3a* cluster are constitutively expressed (Figure 5), even in unchallenged plants. It is unclear whether these paralogues are capable of interacting with unknown elicitors or whether they are just relics of a recent 'birth-and-death' process (Michelmore and Meyers, 1998). Given the fitness cost of *R* gene expression (Tian *et al.*, 2003), unnecessary *R* genes should undergo a 'death' process. We also found many truncated paralogues, mutated via frame-shift (data not shown), or inserted by a retroelement (Figure 2d). The weaker expression of *I2GA-SH23-3* (Figure 5) might suggest that mutations accumulated in the promoter region can lead to a lower expression and thus eventually to the 'death' of the gene. Alternatively, the expressed paralogous proteins may play a role through heteroduplex formation with R3a, as inter- and intramolecular interactions between R protein domains may function as activation switches upon recognition of Avr elicitors (Moffett *et al.*, 2002).

Plants cannot move to escape their microbial environment. To combat disease, plants develop a sophisticated innate immunity system, where *R* genes play a central role (Dangl and Jones, 2001). Comparative genomics may provide insight into how diseases have resulted in differential evolution of *R* loci between closely related plant species. The potato *R3* region has undergone a significant physical expansion compared to the syntenic tomato *I2* region. This expansion may have resulted in functional diversification. For instance, at least 10 *I2*GAs have been found at the *R3b* region (S. Huang, H. Kuang, E. van der Vossen, V. Vleeshouwers, E. Jacobsen, B. Baker and R. Visser, unpublished data), suggesting *R3b* could also be an *I2*GA. The contrasting evolutionary fates of the ancient *I2-R3* complex locus in the closely related tomato and potato genomes are consistent with the opposite evolutionary potentials of the interacting pathogens (McDonald and Linde, 2002). *Fusarium oxysporum* f. sp. *lycopersici* is a soilborne fungus with low genotype diversity, whereas the late blight pathogen *P. infestans* is notorious for its ability to move and mutate (McDonald and Linde, 2002). The great evolutionary potential of *P. infestans* may have stimulated the interacting *R3* complex locus to expand its *R* gene repertoire, as supported by the observed physical expansion and the fact that two functionally distinct genes locate in the locus (Huang *et al.*, 2004). *Phytophthora infestans* also infects tomato, but genomic regions controlling late blight resistance show very limited overlap between tomato and potato (Brouwer *et al.*, 2004; Gebhardt and Valkonen, 2001; Grube *et al.*, 2000). Late blight resistance in the two closely related species is likely conferred by different loci that have evolved independently.

Plant disease resistance genes display two distinct evolutionary patterns contrasting at the rate of evolution (Kuang *et al.*, 2004). The late blight *R* gene *RB*/*Rpi-blb1* from *Solanum bulbocastanum* belongs to the type II (slow evolving) *R* gene class (Song *et al.*, 2003). However, the occurrence of sequence exchanges between paralogues (Figure S2), the multi-allelic nature of the locus (see below), high nucleotide identities between homologues (90–94%) and obscure allelic/orthologous relationships between the SH *R3* haplotype and other *S. demissum* haplotypes (H. Kuang, S. Huang, X. Wang, R. Visser and B. Baker, unpublished data) lead to the putative classification of *R3a* into the type I (fast evolving) class. It remains unknown why these two late blight *R* genes differ by the rate of evolution, but there are some hints. *RB/Rpi-blb1* is resistant to all tested races of *P. infestans* and provides resistance by reducing the infection rate (Song *et al.*, 2003; van der Vossen *et al.*, 2003), while *R3a* is a race-specific gene and displays a typical hypersensitive necrosis response (Huang *et al.*, 2004). *Solanum bulbocastanum* often grows under quite dry conditions (Hawkes, 1990), suggesting the absence of co-evolution with *P. infestans*, whereas *S. demissum* has co-evolved with the pathogen on the cool mountain forests in Toluca Valley of Mexico (Rivera-Pena, 1990), which was recognized as a center of diversity for *P. infestans*.

An intriguing question is why *S. demissum* appears to contain only easily broken *R* genes (Wastie, 1991) but displays durable resistance at the population level (Rivera-Pena, 1990). The polymorphism of parasite recognition capacity in a host population will restrict most isolates of the parasite to grow on most hosts (Hamilton *et al.*, 1990). Allelism is an efficient way of creating recognition polymorphism (Bergelson *et al.*, 2001), and we are currently investigating the multiple allelism of the *R3* complex locus. (S. Huang, V. Vleeshouwers, E. Jacobsen and R. Visser, unpublished data), which might be a natural mechanism of *S. demissum* to suppress late blight epidemics, similar to the concept of *R*-gene polycultures or multilines (Jones, 2001; Mundt, 2002). The isolation of *R3a* and characterization of the *R3* complex locus will facilitate the cloning of other

alleles and thus provide a platform to test the *R*-gene polyculture concept in late blight disease management in commercial potato production.

Experimental procedures

Plant material

The F_1 population of SH83-92-488 (SH) × RH89-039-16 (RH) that segregates for both *R3a* and *R3b* was used for genetic mapping (Huang *et al.*, 2004). SW8540-025 is a recombinant that only carries *R3a*. The susceptible potato clones 1029-31 and cv. Desiree were used for genetic transformation.

Phytophthora infestans *isolates and inoculation*

Phytophthora infestans isolates 89148-09 (*Avr3a*, *Avr3b*), IPO-0 (*Avr3a*, *avr3b*), H30P04 (*avr3a*, *Avr3b*), and 90128 (*avr3a*, *avr3b*) were inoculated on *in vitro* plantlets (S. Huang and V. Vleeshouwers, unpublished data) or detached leaves (Huang *et al.*, 2004).

Genetic mapping

A total of 3000 SH × RH progeny were screened for recombinations in the *R3a* region using the flanking markers cLET5E4 and CT120 (Table 1A). The recombinants were used to determine the position of the *R3a* markers (Figures 1 and 2a). DNA isolation and analysis of molecular markers were described previously (Huang *et al.*, 2004).

Physical mapping

A 10-genome equivalent BAC library of the resistant parent SH, stored in 255 384-well microtiter plates, was screened with the *R3a* markers stepwise: first, positive superpools (1 superpool = 8 BAC pools) were identified; secondly, positive pools were identified from those positive superpools. Plasmid DNA was isolated using the standard alkaline-lysis protocol from pooled bacteria to produce 255 BAC pools. Screening of the BAC pools and identification of single positive BACs were performed as described previously (Rouppe van der Voort *et al.*, 1999).

Plasmids of single BACs were purified using MIDI-PREP columns (Qiagen, Hamburg, Germany). Sequences generated through BAC-end sequencing with SP6 or T7 primers were employed to design PCR primers to develop BAC end markers. The BAC end markers were named according to the BAC pool number followed by S (SP6) or T (T7) (Table 1B). In view of the repetitive nature of the *R3* complex locus, the overlap of two BACs was verified by: (1) *Hin*dIII restriction mapping, (2) non-selective AFLP fingerprinting using *Hin*dIII and *Mse*I/*Taq*I (Simons *et al.*, 1998), and (3) analysis of BAC end markers through T_m-gradient PCR, restriction with 24 frequent cutters, and sequencing of the PCR products. The length of overlapping between two BACs was determined by adding the sizes of co-migrating *Hin*dIII restriction fragments.

DNA sequencing and analysis

The DNA sequences of the BAC clones SH194C17 and SH23G23 (Figure 2d) were determined by using a shotgun sequencing strategy (van der Vossen *et al.*, 2000). Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997) and GENEMARK (Lukashin and Borodovsky, 1998). Multiple sequence alignments were conducted by using CLUSTALX 1.81 (Thompson *et al.*, 1997) and IPS (Parniske *et al.*, 1997) were determined. Diversifying selection was investigated using PAML (Yang, 1997; Yang *et al.*, 2000). Models M7 and M8 in program 'codeml' of PAML were run for all *I2*GAs at the *R3a* cluster. Diversifying selection was confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang *et al.*, 2000). Selection in each site of the *R3a* homologues was calculated using model M8.

Subcloning and transformation of I2*GAs*

Candidate *I2*GAs were subcloned into the binary vector pBINPLUS (van Engelen *et al.*, 1995) as described previously (van der Vossen *et al.*, 2003). Binary plasmids harboring the candidate genes were transformed to *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991) and introduced into 1029-31 and cv. Desiree according to standard protocols (Visser *et al.*, 1991).

Transcript analysis of the R3a *cluster*

Total RNA was extracted from detached leaves of SH inoculated with *P. infestans* IPO-0, 90128, and water using TRIZOL (Invitrogen, Carlsbad, CA, USA). The mRNA was isolated from the total RNA samples using the OLIGOTEC kit (Qiagen). The 5′- and 3′-ends of the *R3a* transcript were determined by rapid amplification of cDNA ends (RACE) using the MARATHON kit (BD Bioscience, USA) with nested gene-specific primers (3RACE4-6 and 5RACE4-6, Table 1D). The cDNA for 5′ RACE was synthesized with the primer 5RACE0. PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). For gene-specific RT-PCR (Table 1E), an aliquot of 0.1 μg mRNA from each sample was used to generate single-stranded cDNA using SUPERSCRIPT II (Invitrogen). Actin primers were used to monitor mRNA concentration (Table 1E). Genomic DNA of SH and RH and BAC subclones were used as control. Gene-specific amplification was confirmed by sequencing.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2365/TPJ2365sm.htm

Figure S1. The distribution of codons under diversifying selection in *R3a*.

Figure S2. Sequence relationships between members of the *R3a* cluster.

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