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ORIGINAL PAPER

Fine mapping of the *Ph-3* gene conferring resistance to late blight (*Phytophthora infestans*) in tomato

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Abstract Late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is a devastating disease for tomato and potato crops. In the past decades, many late blight resistance (*R*) genes have been characterized in potato. In contrast, less work has been conducted on tomato. The *Ph-3* gene from *Solanum pimpinellifolium* was introgressed into cultivated tomatoes and conferred broad-spectrum resistance to *P. infestans*. It was previously assigned to the long arm of chromosome 9. In this study, a high-resolution genetic map covering the *Ph-3* locus was constructed using an F_2 population of a cross between *Solanum lycopersicum* CLN2037B (containing *Ph-3*) and *S. lycopersicum* LA4084. *Ph-3* was mapped in a 0.5 cM interval between two markers, Indel_3 and P55. Eight putative genes were found in the corresponding 74 kb region of the tomato Heinz1706 reference genome. Four of these genes are resistance gene analogs (RGAs) with a typical nucleotide-binding adaptor shared by APAF-1, *R* proteins, and CED-4 domain. Each RGA showed high homology to the late blight *R* gene *Rpi-vnt1.1* from *Solanum venturii*. Transient gene silencing indicated that a member of this RGA family is required for *Ph-3*-mediated resistance to late blight in tomato. Furthermore, this RGA family was also found in the potato genome, but the number of the RGAs was higher than in tomato.

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Introduction

Late blight (LB), caused by the oomycete pathogen *Phytophthora infestans*, is considered as a threat to global food security (Gregory et al. 2009). It is one of the most devastating diseases for cultivated tomatoes (*Solanum lycopersicum*) and potatoes (*Solanum tuberosum*) worldwide (Foolad et al. 2008). In 2007, epidemics of LB caused the loss of approximately 638,900 tons of processing tomato production in China's main growing area, Inner Mongolia (Li 2008). Meanwhile, this disease also threatened Florida's winter tomato production, a $464 million industry that accounted for 36 % of American production of fresh tomatoes in 2007 (Schultz et al. 2010). The only efficient way to protect tomato and potato crops from LB is by the application of chemicals. However, fungicide-resistant strains of the pathogen have emerged; therefore, it is increasingly difficult to control this disease (Fry and Goodwin 1997a, b; Goodwin et al. 1998). Breeding for LB resistance is an economical and environmentally friendly strategy that provides an attractive alternative to chemical control. Various levels of LB resistance exist in wild relatives of cultivated plants, which can be used as potential resources for breeding crops with LB resistance.

In potato, introgression of *R* genes from germplasm has been carried out over the last century. To date, more than 30 major or qualitative LB *R* genes have been identified from diverse *Solanum* species, and some of these *R* genes have been cloned [reviewed by Hein et al. (2009) and Vleeshouwers et al. (2011)]. In addition, numerous quantitative trait loci (QTLs) have been identified from cultivated and wild potato species (Gebhardt and Valkonen 2001; Ghislain et al. 2001; Tan et al. 2008). In some cases, qualitative and quantitative resistances are hard to distinguish and could in fact be caused by the same genes (Rauscher et al. 2010; Rietman et al. 2012). In tomato, both qualitative and quantitative LB resistances have been reported. Three major LB resistance genes, *Ph-1*, *Ph-2* and *Ph-3*, were identified in the wild species *Solanum pimpinellifolium* (Bonde and Murphy 1952; Gallegly and Marvel 1955; Peirce 1971; Moreau et al. 1998; Chunwongse et al. 2002). The *Ph-1* gene maps to chromosome 7 and confers resistance only to *P. infestans* race T_0 (Bonde and Murphy 1952; Gallegly and Marvel 1955; Peirce 1971). The *Ph-2* gene, conferring incomplete LB resistance, was identified in *S. pimpinellifolium* line WVa 700 and is located on the distal part of the long arm of chromosome 10 (Gallegly and Marvel 1955; Moreau et al. 1998). Resistance conferred by *Ph-1* and *Ph-2* was overcome by different *P. infestans* isolates from Taiwan, Indonesia, Nepal and The Philippines (AVDRC 1995, 1998, 1999). This prompted further screening of tomato germplasm for new LB resistance genes. As a result, *S. pimpinellifolium* L3708 was found to be highly resistant to a wide range of *P. infestans* isolates that overcome *Ph-1* and *Ph-2*-related resistance (Black et al. 1996a, b). Genetic study indicated that LB resistance in L3708 was conditioned by a single partially dominant gene, *Ph-3*, which was mapped to the long arm of chromosome 9 (Black et al. 1996a; Chunwongse et al. 2002). In addition, Foolad et al. (2006, 2008) reported a new *S. pimpinellifolium* accession (PI270443), which exhibited strong resistance to multiple *P. infestans* isolates. Recently, two genomic regions on chromosome 1 and 10 were demonstrated to govern the resistance derived from this accession (Merk et al. 2012). Despite the different chromosomal locations, the resistance of PI270443 was similar to that of the tomato breeding lines containing either *Ph-3* or a combination of *Ph-2* and *Ph-3* when inoculated with an aggressive *P. infestans* isolate that belongs to the US-13 clonal lineage (Merk et al. 2012). Thus, the possibility remains that the PI270443 resistance on chromosome 10 is an allele of the *Ph-2* gene (Merk et al. 2012). Breeding efforts to transfer this resistance to elite tomato lines are underway (Merk and Foolad 2012). In addition to the *Ph* genes mentioned above, QTLs conferring race-non-specific resistance have been identified from *Solanum pennellii* and *Solanum habrochaites* (Smart et al. 2007; Brouwer et al. 2004; Brouwer and St Clair 2004; Li et al. 2011b. However, these QTL effects are relatively small and vulnerable to the environment. Occasionally, the QTLs are linked with some undesirable horticultural traits, such as reductions in yield and fruit size (Brouwer and St Clair 2004). Therefore, using them in practical plant breeding programs may not be advisable.

Thus far, *Ph-2* and *Ph-3* have been widely used in the tomato breeding programs for LB resistance (Moreau et al. 1998; Chunwongse et al. 2002; Foolad et al. 2008; Gardner and Panthee 2010; Panthee and Gardner 2010). Stacking of *Ph-2* and *Ph-3* confers strong resistance in the field (Gardner and Panthee 2010; Panthee and Gardner 2010). The *Ph-3* gene is considered the most effective source of LB resistance in tomato (Chunwongse et al. 2002; Kim and Mutschler 2006). However, no further research has been conducted on this widely used LB resistance gene in tomato.

The objective of this study is to fine map the *Ph-3* gene. Using a segregating F_2 population (*Solanum lycopersicum* CLN2037B X *S. lycopersicum* LA4084), we mapped *Ph-3* to a 74 kb region of the tomato reference genome, which harbors an RGA cluster with high homology to the late blight *R* gene *Rpi-vnt1.1* from potato. Further functional analysis with virus-induced gene silencing (VIGS) demonstrated that *Ph-3* is a member of this RGA family.

Materials and methods

Plant materials

The resistant line *S. lycopersicum* CLN2037B containing the *Ph-3* gene (kindly provided by the Asian Vegetable Research and Development Center, AVRDC) was crossed with the susceptible line *S. lycopersicum* LA4084 (kindly provided by the Tomato Genetics Resource Center, TGRC). The resulting F_1 plants were self-crossed and F_2 seeds were bulked. A total of 861 F_2 plants were used for inheritance studies and genetic mapping of the *Ph-3* gene. Subsequently, another 1,033 F_2 individuals were subjected to a recombinant screening using markers P31 and P60, flanking the *Ph-3* locus. The selected F_2 recombinants were tested for late blight resistance. To further confirm the phenotype, 1,044 F_3 plants derived from 31 F_2 recombinants were evaluated for LB resistance.

DNA extraction and marker development

Genomic DNA was extracted from fresh leaves of 2-week-old tomato seedlings using the Cetyl Trimethyl Ammonium Bromide method (Fulton et al. 1995). To construct the genetic map around the *Ph-3* locus, molecular markers

from the long arm of chromosome 9 (http://solgenomics.net/) were selected and used to screen the parental lines for polymorphisms. To increase the map resolution, a series of cleaved amplified polymorphic sequences (CAPS) and simple-sequence repeat (SSR) markers were designed using the publicly available tomato genome sequence (http://solgenomics.net/). Information on the primers for the identified markers is listed in Table S1.

Disease assay

An isolate of *P. infestans* race $T_{1,2,4}$, which is virulent to *Ph-1* and *Ph-2*, but not to *Ph-3*, was used in LB disease assays (Feng et al. 2004). The isolate was maintained in 15 % dimethyl sulfoxide solution at −80 °C and propagated on rye sucrose agar medium in the dark at 19 °C for 15–20 days before inoculation.

The whole-plant assay was performed as described by Chen et al. (2009) and Brouwer et al. (2004). In brief, plants with five fully expanded leaves were inoculated using a paint sprayer to disperse the suspension (1,000 sporangia/ml) over the plants. Inoculated plants were incubated at 100 % relative humidity (RH) and 20 ± 2 °C without light for the first 24 h. Thereafter, plants were grown at 70–90 % RH and 20 ± 2 °C with a 12 h light period.

Disease severity (DS) was rated at 7–10 days post inoculation (DPI) on a scale of 0–6. 0 = no symptoms; 1 = 1–5 % of leaf area affected and showing small lesions; 2 = 6–15 % of leaf area affected and showing restricted lesions; 3 = 16–30 % of leaf area affected and/or showing water-soaked flecks on stems; 4 = 31–60 % of leaf area affected and/or with a few stem lesions; 5 = 61–90 % of leaf area affected and/or with expanding stem lesions; 6 = 91–100 % of leaf area affected and/or with extensive stem damage, or the most heavy disease severity resulting in dead plants. Two categories were assigned to all tested plants based on the score: resistant (0–4) and susceptible (5–6).

Linkage analysis and genetic mapping

The genetic linkage map was constructed using JoinMap 4 (Van Ooijen 2006) with a minimum logarithm of odds (LOD) threshold of 3.0. The Kosambi mapping function (Kosambi 1944) was used to convert recombinant frequencies to map genetic distances in centi-Morgans (cM). MapQTL 4.0 (Van Ooijen and Maliepaard 1996) was used to perform the QTL analysis.

Gene prediction and sequence analysis

The online program FGENESH was used to predict open reading frames (ORFs) in the target region (http://linux1.softberry.com/). Protein function was predicted with the InterProScan program (http://ebi.ac.uk/Tools/InterProScan/) and the results were compared with the annotations from the International Tomato Annotation Group (ITAG). ClustalW2 was used to align multiple sequences (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Statistical analysis

The Chi-square test for goodness of fit was performed to test for deviations of observed and expected segregation rations with SAS 8.0 (http://v8doc.sas.com/sashtml/).

Virus-induced gene silencing (VIGS)

The VIGS experiments were performed as described by Liu et al. (2002). To make the VIGS constructs, primer pairs (Fig. S1) were designed based on Heinz1706 RGA sequences to amplify fragments from the cDNAs of the LB-resistant line CLN2037B. PCR products were cloned into the Gateway-compatible vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) and subsequently recombined into tobacco rattle virus-based VIGS vector pTRV2 (Liu et al. 2002). The pTRV2 vectors carrying the RGA fragments were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. A 100 ml culture of each *A. tumefaciens* clone was grown overnight at 28 °C in YEP medium (1000 ml YEP containing 5 g beef extract, 5 g peptone, 5 g sucrose, 1 g yeast extract and 2 ml 1 M $MgSO_4$) with antibiotics (50 mg/ml kanamycin and 50 mg/ml rifampicin). The cells were resuspended in infiltration medium MMA (1,000 ml MMA containing 20 g sucrose, 5 g MS salts, 1.95 g MES and 1 ml 200 mM acetosyringone, pH = 5.6) till $OD_{600} = 2$. Cultures were kept at room temperature for 1–6 h before agroinfiltration. *Agrobacterium* strains containing the pTRV1 vector and pTRV2 were mixed at a 1:1 ratio and co-infiltrated into the cotyledons of 10-day-old tomato seedlings of the LB-resistant line CLN2037B (harboring *Ph-3*) and M82 (susceptible control). The tomato phytoene desaturase gene (*tPDS*) amplified from cDNA of CLN2037B was used as the reference gene to assess the VIGS system. The pTRV2 empty vector (pTRV2-ev) and water were used as negative controls. Forty days after the agroinfiltration, the plants were inoculated with *P. infestans*.

Ten plants were used for the infiltration using constructs pTRV2-tPDS, pTRV2-ev, and water, while 35 plants were used for pTRV2-Ph3V1 and pTRV2-Ph3V2. A few plants died after agroinfiltration. Tomato plants were grown in pots at 23 ± 2 °C in the greenhouse. The VIGS experiments were performed twice.

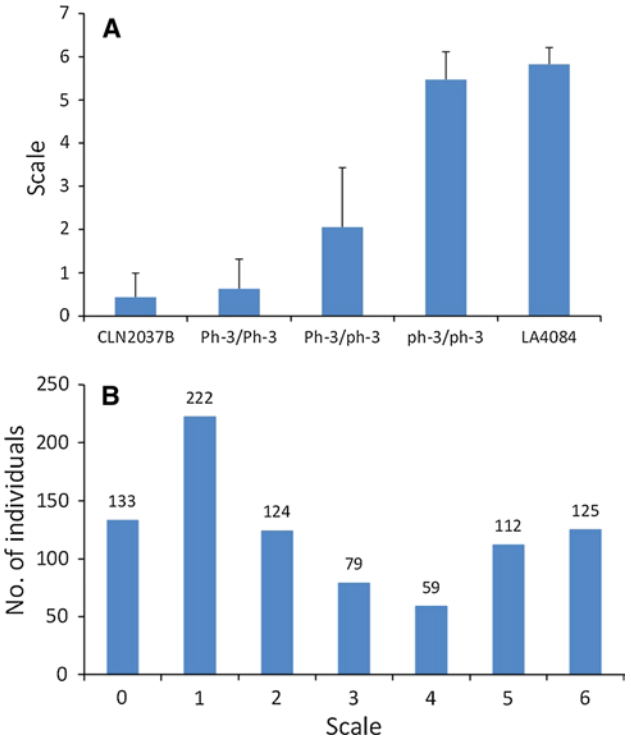

Fig. 1 Disease assays on the parental lines and the F_2 population. **a** The DS of parental lines CLN2037B and LA 4084, and average DS of homozygous (Ph-3/Ph-3) or heterozygous plants (Ph-3/ph-3) at the *Ph-3* locus or plants not containing *Ph-3* (ph-3/ph-3), using the flanking markers P31 and P60 as an indicator of the presence or absence of the *Ph-3* allele; **b** The frequency distribution of disease scales in the F_2 population. The *numbers above the bars* indicate the number of individuals for each scale

Results

Ph-3 is partially dominant

Previously, *Ph-3* has been described as a partially dominant gene for LB resistance (Black et al. 1996a, b; Chunwongse et al. 2002). To verify this conclusion, we performed the LB assay on a segregating F_2 population and their parental lines. The resistant parent CLN2037B exhibited a high level of LB resistance to *P. infestans* isolate $T_{1,2,4}$, while the other parent, LA4084, was fully susceptible (Fig. 1a). Of the 861 F_2 individuals (seven plants were excluded because of infection with other diseases), 237 were completely or extensively blighted already at 7 DPI (showing DS levels between 5 and 6) and were regarded as susceptible. The remaining 617 plants showed DS levels from 0 to 4 and were considered as resistant (Fig. 1b). The segregation between resistant (R) and susceptible (S) plants was in agreement with a 3R:1S segregating ratio ($\chi^2_{3:1} = 3.45$, $P = 0.06$), suggesting the presence of a single dominant resistance gene. To confirm the partial dominance of *Ph-3*, we analyzed the homozygous and heterozygous plants containing the *Ph-3* gene. Plants without recombination at the *Ph-3* locus (as determined by flanking markers P31 and P60) were used for this analysis. Among them, the plants not containing the *Ph-3* gene were susceptible to *P. infestans* (mean DS = 5.47) (Fig. 1a). In contrast, homozygous F_2 individuals containing the *Ph-3* locus were highly resistant, with DS levels ranging from 0 to 2 (mean DS = 0.63). The heterozygous plants showed intermediate DS levels ranging from 0 to 4 (mean DS = 2.05) (Fig. 1a). Hence, the *Ph-3* gene also showed partial dominance in our population.

Fine mapping of the *Ph-3*

For fine mapping of the *Ph-3* gene, 21 markers (Table S1), covering a 1.79 Mb interval on tomato chromosome 9, were developed. All 21 markers showed a linear order between their genetic and physical locations. Nineteen of the markers (except TES0562 and T0156) were mapped to 16 loci with an average interval of 0.4 cM. Considering that *Ph*-3 is a partially dominant gene, QTL analysis was firstly performed using the 861 F_2 individuals to exclude the possibility that there were other QTL effects in this region. The results showed only a single peak, with a LOD score of 3.88 explaining 93.56 % of the phenotypic variance (Fig. 2a). We therefore concluded that the *Ph-3* gene mapped within a genetic interval of 0.3 cM on the long arm of chromosome 9. The genetic distances between *Ph-3* and the closest flanking markers, Indel_3 and RGA2M1, were 0.2 and 0.1 cM, respectively (Fig. 2b).

The phenotype of some important recombinant plants could not be confirmed because of the absence of F_3 seeds. Therefore, we further confirmed the map position of the *Ph-3* gene in a second set of recombinants. Another 1,033 F_2 individuals derived from the same cross (CLN2037B × LA4084) were screened for recombinants between markers P31 and P60, and 31 recombinants were found. These plants were analyzed with six other markers between P31 and P60 (Fig. 2c). The F_3 families of the 31 selected F_2 recombinants were subsequently tested for late blight resistance (Table 1). Of them, 30 F_3 families showed either a consistent phenotype (all plants are resistant/susceptible) or Mendelian inheritance (segregating for resistance according to a single dominant gene model). By combining the F_2 genotypes and the phenotypes of their corresponding F_3 families, the *Ph-3* gene was mapped between markers Indel_3 and P55 (Table 1). For example, the F_2 recombinant B212 was heterozygous for marker alleles downstream of the marker Indel_3 and LB resistance segregated in its F_3 family, thus it was deduced that the resistance gene is downstream of Indel_3 (Table 1). From the five nearest upstream recombinants (B212,

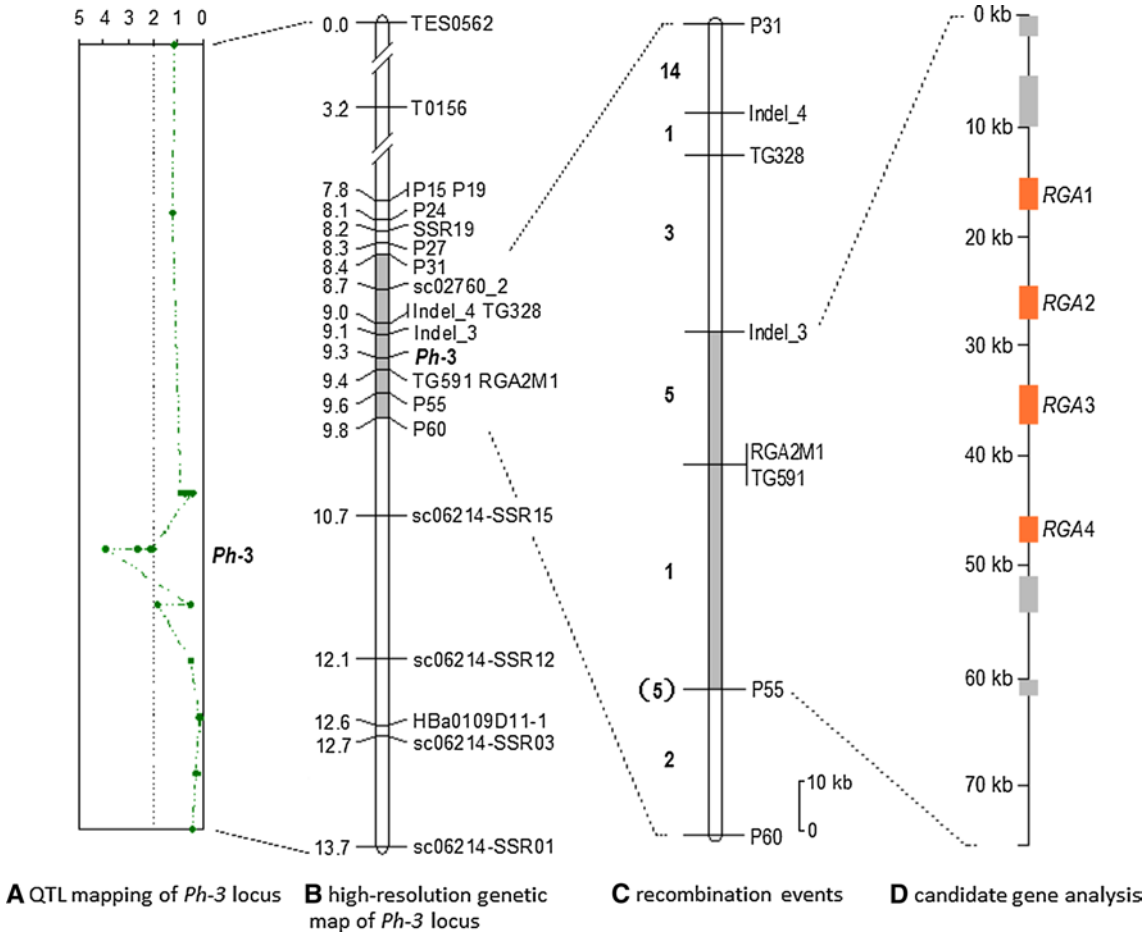

Fig. 2 Genetic and physical maps of the *Ph-3* gene and candidate genes analysis. **a** QTL mapping of the *Ph-3* gene. *Numbers on top of the graph* are LOD values. **b** A high-resolution genetic map of the *Ph-3* locus. Positions of the markers are indicated in cM. The linkage map was generated using 861 F_2 individuals using JionMap 4.0. **c** Distribution of recombination events over the physical map between markers P31 and P60. These recombinants were screened from another 1,033 F_2 individuals. *The numbers on the left of the bar* indicate the number of recombinant plants identified between the two markers. P55 is a dominant marker, thus the recombination sites of five plants (between brackets) were uncertain. **d** The location of four NBS-type RGAs named as *RGA1*, *RGA2*, *RGA3* and *RGA4* in the Heinz1706 genome sequence at the *Ph-3* locus

N1036, N1200, N337 and N1384) and one downstream recombinant (N299), it was concluded that the *Ph-3* gene is located between markers Indel_3 and P55. In the F_3 family of B247, unexpectedly, one resistant plant was found. We considered this an escape and this family was disregarded for fine mapping.

Analysis of the candidate gene family in the *Ph-3* region

Based on the linkage map, the genomic region of CLN2037B between Indel_3 and P55 contains the *Ph-3* gene. These two markers are located on one Heinz1706 BAC, C09HBa0165P17, and are 74 kb apart. According to the tomato genome annotation (ITAG2.4 version), eight putative protein-coding genes were predicted between these markers: a chaperone protein DnaJ, a transferase, an RNA binding protein-like protein, an NAD-dependent epimerase, and four clustered NBS-type resistance proteins (Fig. 2d). To distinguish these four putative RGAs from each other, they were named as *RGA1*, *RGA2*, *RGA3* and *RGA4* according to their order on the physical map, counting from marker Indel_3. Sequence identity analysis indicated that these four RGAs were closely related to the *Tomato mosaic virus*-resistant gene *Tm-2*^{2} and potato late blight resistance gene *Rpi-vnt1.1* (Table 2). *Tm-2*^{2} and *Rpi-vnt1.1* share 75 % amino acid identity and both are located on the long arm of chromosome 9 in tomato and potato (Lanfermeijier et al. 2003; Foster et al. 2009; Pel et al. 2009). To date, all published LB resistance genes in potato contain a nucleotide-binding site–leucine-rich repeat (NBS–LRR) domain, thus we focused on these four RGAs for functional analysis.

Table 1 Disease tests on F_3 families of the 31 selected recombinants

F_2 recombinants	Genotype (F_2)[a]							Phenotype (F_3 progeny)[b]	
No.	P31	Indel_4	TG328	Indel_3	RGA2M1	P55	P60	R	S
N605	a	h	h	h	h	d	h	34	14
N116	h	b	b	b	b	b	b	12	0
N264	h	b	b	b	b	b	b	12	0
N747	h	b	b	b	b	b	b	17	0
N848	h	b	b	b	b	b	b	17	0
N81	h	h	b	b	b	b	b	12	0
B481	a	a	a	h	h	d	h	44	23
B212	a	a	a	a	h	d	h	38	23
N1036	a	a	a	a	h	d	h	34	14
N1200	h	h	h	h	b	b	b	46	0
N299	h	h	h	h	h	b	b	39	8
N1225	h	h	h	h	h	d	b	38	8
N872	h	h	h	h	h	d	b	34	13
N1183	a	a	a	a	a	d	h	0	12
N72	a	a	a	a	a	d	h	0	12
N1100	b	b	b	b	b	b	h	9	0
N588	b	b	b	b	b	b	h	12	0
N734	h	h	h	h	h	d	a	33	10
N337	b	b	b	b	h	d	h	34	14
N1384	h	h	h	h	a	d	a	0	22
N1097	b	b	b	h	h	d	h	34	11
B247	h	h	h	a	a	d	a	1	51
N247	h	a	a	a	a	d	a	0	12
N635	h	a	a	a	a	d	a	0	24
N889	h	a	a	a	a	d	a	0	29
N953	h	a	a	a	a	d	a	0	24
N1065	b	h	h	h	h	d	h	34	13
N1087	b	h	h	h	h	d	h	34	12
N27	b	h	h	h	h	d	h	22	10
N762	b	h	h	h	h	d	h	38	7
B522	b	h	h	h	h	d	h	23	27
CLN2037B	b	b	b	b	b	b	b	60	0
LA4084	a	a	a	a	a	a	a	0	54

[a] Genotypes of recombinant F_2 individuals, *a* homozygous like the susceptible parent LA4084, *b* homozygous like the resistant parent CLN2037B, *h* heterozygous, *d* either a or h

[b] The number of resistant or susceptible plants. R indicates the plant has a DS score of 0–4 and is considered as resistant. S indicates the plant has a DS score of 5–6 and is regarded as susceptible

Functional analysis of the role of the candidate gene family in resistance conferred by *Ph-3*

To test the potential involvement of members of the *Tm-2*^{2} family in *Ph-3* LB resistance, a transient gene silencing approach through VIGS was used to perform loss of function analysis. Two conserved regions of these four RGAs were selected and used to construct the VIGS vectors (Ph3V1 and Ph3V2), which could simultaneously silence all four RGAs (Supplemental Fig. S1). To check the specificity of the VIGS constructs, Ph3V1 and Ph3V2 were used as query sequences in a BLAST search against the tomato whole genome sequence. In total, five hits were identified, including the four predicted RGAs in the target region and the *tomato mosaic virus*-resistance gene *Tm-2*^{2}. Based on the tomato genome sequence information from the tomato genome annotation (ITAG2.4 version), the *Tm-2*^{2} gene is 53 Mb from the predicted RGAs region. Moreover, tomato

Table 2 Sequence identity at the amino acid level among the four RGAs in the *Ph-3* gene region, as well as their identities with the *Tm-2²* gene and the *Rpi-vnt1.1* gene

	RGA2	*RGA3*	*RGA4*	*Tm-2²*	*Rpi-vnt1.1*
RGA1	90.0 %	77.0 %	79.0 %	67.0 %	68.0 %
RGA2		77.0 %	79.0 %	67.0 %	69.0 %
RGA3			95.0 %	71.0 %	74.0 %
RGA4				74.0 %	78.0 %
Tm-2²					75.0 %

lines carrying only *Tm-2²* were susceptible to *P. infestans* $T_{1.2.4}$ (our unpublished data), indicating that the *Tm-2²* was not involved in LB resistance.

Two weeks after agroinfiltration, the *PDS*-silenced plants exhibited a photo-bleached phenotype, indicating a successful silencing effect. Upon inoculation with *P. infestans*, all M82 plants infiltrated with the empty pTRV2 construct showed pathogen sporulation; however, no sporulation was observed on CLN2037B plants infiltrated with the same vector (Fig. 3). This suggested that the tobacco rattle virus infection did not alter the pathogenesis of *P. infestans* on tomato, nor did it affect *Ph-3* mediated resistance. In contrast, sporulation of *P. infestans* was observed on CLN2037B plants infiltrated with pTRV2-Ph3V1 in 21 out of 29 plants. Also, 24 out of 31 CLN2037B plants that were agroinfiltrated with pTRV2-Ph3V1 were susceptible to *P. infestans*. Two independent VIGS experiments were performed and the results were consistent. Combined with our fine mapping data, these results strongly suggested that members of the *Tm-2* family of RGAs were involved in *Ph-3* mediated resistance.

Microsynteny comparison of the genomic region around *Ph-3* between tomato and potato

Using the whole genome sequences of tomato and potato, we compared the *R* gene cluster around the *Ph-3* locus between these two closely related species. The 74 kb tomato genome sequence (between markers Indel_3 and P55) aligned with a 113 kb homologous region of the potato genome. As shown in Fig. 4, both genomes were highly collinear, except for the interval covering these RGAs (*RGA1–RGA4* in tomato and *RGA1p–RGA8p* in potato). Within this interval, both the number and structure of the *R* genes were different. In tomato, there were four complete RGAs with single ORFs in the *Ph-3* cluster, but the corresponding region in the potato genome comprised six complete RGAs and two partial RGAs (*RGA4p* and *RGA6p*; Fig. 4), which did not contain LRR-encoding domains.

Discussion

The *Ph-3* gene confers partial dominant resistance to *P. infestans*

Plant resistance responses against pathogens are traditionally classified as race-specific, race-non-specific, and

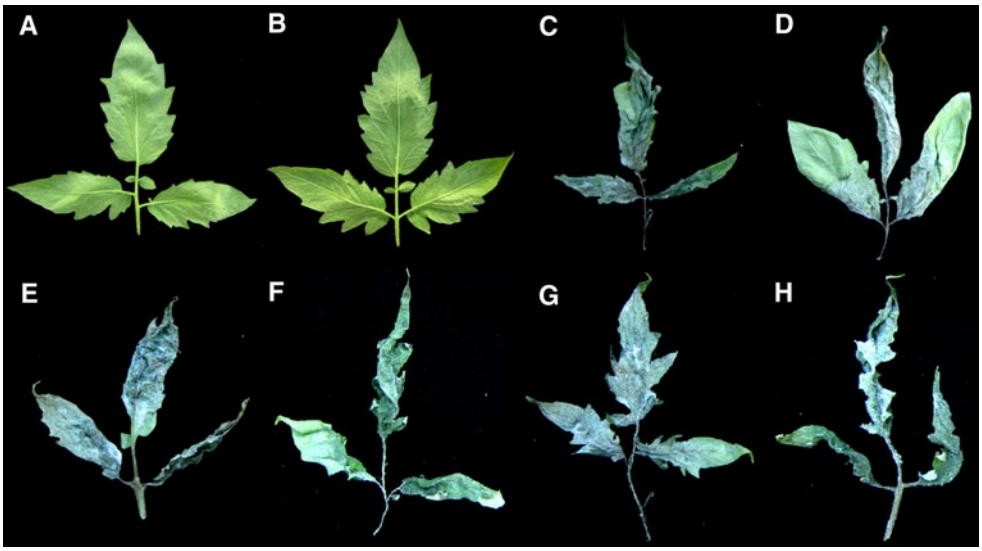

Fig. 3 Leaves of plants inoculated with *P. infestans* in virus-induced gene silencing experiments. Nine days after inoculation, no symptoms of fungal infection were visible on the abaxial side of leaves of tomato line CLN2037B infiltrated with water (**a**) or pTRV2-ev (**b**), while heavy sporulation was detected on the leaves of pTRV2-Ph3V1 (**c**) or pTRV2-Ph3V2 (**d**) treated CLN2037B plants. Clear sporulation was observed on leaves of the susceptible control M82 infiltrated with water (**e**), pTRV2-ev (**f**), pTRV2-Ph3V1 (**g**) or pTRV2-Ph3V2 (**h**)

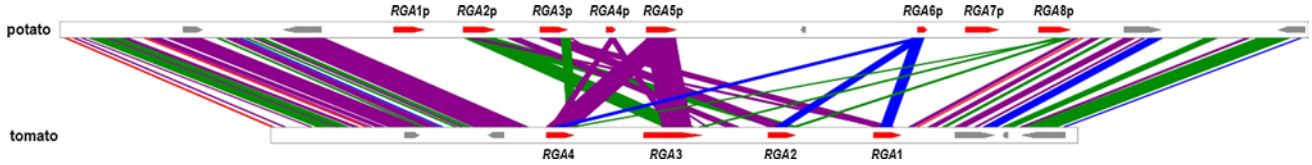

Fig. 4 Microsynteny of the *Ph-3* region between tomato and potato. The 74 kb tomato genome sequence (*bottom*) between marker Indel_3 and P55 is aligned with the homologous region of the potato genome (*top*). The *red arrows* show the predicted *R* genes with their orientation. The *red lines* linking the tomato and potato sequences indicate that the identity of both genomes is above 95 % in this region; *blue lines* indicate identity of 90–95 %; *purple lines* indicate identity of 85–90 %; and *green lines* indicate identity of 80–85 %. The regions with the identity percentages below 80 % are not shown

non-host resistance (Agrios 1997). Typical race-specific resistance is based on the presence of major *R* genes. The *R* genes are supposed to encode specific receptors that, upon perception of their corresponding avirulence (AVR) protein, initiate signal transduction pathways leading to resistance, often associated with a hypersensitive response (HR) (Hammond-Kosack and Jones 1997). Previously, the *Ph-3* gene was characterized as a partially dominant gene, which did not explain 100 % of the observed variation (Chunwongse et al. 2002). In this study, we confirmed the partial dominance of this gene to *P. infestans* isolate $T_{1,2,4}$ using a large F_2 population through a reliable whole-plant assay. We found that the homozygous F_2 plants at the *Ph-3* locus were highly resistant, while the heterozygous plants showed intermediate resistance. Kim and Mutschler (2006) reported similar findings.

The *Ph-3* gene is closely linked with the marker RGA2M1

In a previous study, the *Ph-3* gene was mapped to the long arm of chromosome 9, close to restriction fragment length polymorphism marker TG591A (Chunwongse et al. 2002). Later, two CAPS markers, TG591 and TG328, were used to introduce *Ph-3* into tomato breeding lines (Foolad et al. 2008; Robbins et al. 2010). Through analysis of the corresponding BAC sequences, Robbins et al. (2010) speculated that the likely position for *Ph-3* was between TG328 and TG591. In this study, a large population from a cross between an LB-resistant line, CLN2037B (containing *Ph-3*), and a susceptible parent, LA4084, was used to fine map *Ph-3*. A high-resolution genetic map was constructed using 21 polymorphic markers. Based on the genome sequence of *S. lycopersicum* Heinz1706, the physical distance between markers TES0562 and sc06214-SSR01 was estimated at about 1.79 Mb. The average physical distance per cM in this region was calculated to be 128 kb/cM, which was lower than the average value (172 kb/cM) of tomato euchromatic regions (Kenta et al. 2010). This suggested a relatively high recombination rate at the end of the long arm of chromosome 9 in our F_2 mapping population. 861 F_2 individuals and the F_3 families of the 31 F_2 recombinants selected from another 1,033 F_2 individuals were tested for resistance to *P. infestans*. The *Ph-3* gene was ultimately located between marker Indel_3 and P55. In the initial mapping population of 861 plants, one recombinant (B410) was identified between the resistance gene and the marker RGA2M1. The genotype of B410 was heterozygous at the RGA2M1 locus but it was susceptible to LB. Unfortunately, this plant was seriously infected and no progeny of this recombinant could be maintained for confirmation. Because RGA2M1 is not specific for any of the individual RGA, it is difficult to exclude any of the four RGAs as the candidate of *Ph-3*. Nevertheless, we can conclude that RGA2M1 is closely linked to the *Ph-3* gene.

The *Ph-3* gene belongs to the *Tm-2²/Rpi-vnt1.1* family

Based on the high-resolution map and the tomato genome sequence, the *Ph-3* gene was finally mapped to a 74-kb region in the reference tomato genome. Potato and tomato both belong to the *Solanaceae* family and have highly syntenic genomes (Tanksley et al. 1992). In addition, *R* genes tend to be clustered at co-linear chromosome regions across these two genera (Grube et al. 2000). For instance, the *R3a* LB resistance gene cluster on the long arm of potato chromosome 11 is co-linear with the *I2* locus for resistance to *Fusarium* in tomato (Huang et al. 2005). In addition, the LB resistance gene *Rpi-blb2* from chromosome 6 was found to be a tomato *Mi-1* gene homolog, and both genes shared 82 % similarity at the amino acid level (van der Vossen et al. 2005). Using the Comparative Map Viewer (http://solgenomics.net), we found that on the long arm of chromosome 9, collinearity exists between tomato and potato. Some LB resistance genes have been mapped on potato chromosome 9, including *Rpi-moc1* (also known as *Rpi-mcq1*) (Smilde et al. 2005) and *R8* from *Solanum demissum* (Jo et al. 2011). Furthermore, Golas et al. (2010) identified an LB resistance gene, *Rpi-dlc1*, from *Solanum dulcamara*, which belongs to *subgenus Potatoe*, and mapped the gene within a cluster on the lower arm of chromosome 9.

Although the similarity of the genetic locations is highly suggestive, it remains to be shown if these genes have allelic relationships or whether they have more distant evolutionary relationships associated with different recognition specificities.

Based on genome sequence information and functional analyses, we demonstrated that *Ph-3* was located in or near an *R* gene cluster containing four typical CC-NBS-type RGAs that shared high amino acid identities with *Tm-2*^{2} or *Rpi-vnt1.1* (Table 2). Transient silencing of the candidate RGAs in the resistant tomato line CLN2037B led to loss of resistance to *P. infestans*, suggesting that *Ph-3* belongs to the *Tm-2*^{2}*/Rpi-vnt1.1* family. Among the four RGAs from the reference genome, *RGA1*, *RGA2* and *RGA4* contain one exon, while *RGA3* contains four predicted exons. Comparison of the deduced protein sequences of the four RGAs revealed that the identities among them ranged from 77 to 95 % at the protein level (Table 2). Gene duplication and sequence exchange between *R* gene homologs are major mechanisms that shape *R* gene diversity in plants (Kuang et al. 2004). The high identities indicated that these four RGAs might have a common origin and have arisen through tandem duplication. In addition, we also compared the tomato and potato genome sequence covering the *Ph-3* locus. The number and overall length of RGAs from two genomes were diverse, which is again in agreement with the hypothesis that *R* gene clusters evolve differently from other parts of the genome by local duplications potentially caused by unequal crossovers.

Many genes conferring LB resistance have been cloned from potato relatives (Hein et al. 2009; Vleeshouwers et al. 2011; Li et al. 2011a). In tomato, several LB qualitative resistance genes were discovered but none of them have been cloned yet. The results obtained in this study will not only help to clone the *Ph-3* gene but also will increase our understanding of the evolution of resistance to *P. infestans* in Solanaceous crops. Currently, cloning of *Ph-3* gene is ongoing in our laboratory, which will further increase our understanding of partial resistance and genetic evolution of *R* genes in both tomato and potato. Interestingly, all tomato late blight resistance genes identified to date are derived from the wild tomato species *S. pimpinellifolium*, which thrives in the coastal areas of Peru and Ecuador (Zuriaga et al. 2009). This might hint at a special co-evolution between *S. pimpinellifolium* and *P. infestans* in this geographic region.

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