Fine Mapping of the *Ph-2* Gene Conferring Resistance to Late Blight (*Phytophthora infestans*) in Tomato

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Abstract

Late blight is a devastating tomato disease. Breeding new varieties with multiple resistance (*R*) genes is highly effective for preventing late blight. The *Ph-2* gene mediates resistance to *Phytophthora infestans* race T₁ in tomato. In this study, we used an F₂ population derived from a cross between *Solanum lycopersicum* Moboline (resistant) and LA3988 (susceptible) cultivars for the fine mapping of *Ph-2*. Two flanking markers, CAPS-1 and CC-Ase, mapped *Ph-2* to a 141-kb genomic region

containing 21 projected genes, 5 of which were identified as putative *R* genes. The *Solyc10g085460* coding sequence varied significantly between the parents. The markers developed and candidate genes identified in this study shall be useful for the molecular breeding of tomato exhibiting increased late blight resistance and for the cloning of the *Ph-2* gene.

Keywords: candidate genes, fine mapping, late blight, *Ph-2*, tomato

Tomato (*Solanum lycopersicum* L.) is an important economic crop worldwide. However, late blight epidemics caused by the oomycete *Phytophthora infestans* (Mont.) de Bary have adversely affected the global production of tomato (Ohlson and Foolad 2016). In 2007, late blight epidemics resulted in the loss of approximately 638,900 tons of tomato in Inner Mongolia, China (Li 2008). In 2009, the total tomato yield losses due to late blight reached \$110 million in the United States (Nowicki et al. 2012). Moreover, the financial losses associated with yield reductions and late blight control measures are estimated to exceed \$5 billion annually (Elsayed et al. 2012). Therefore, effective strategies for preventing late blight are required. Outbreaks of late blight can occur and spread rapidly, making them difficult to control. For many years, several fungicides have been widely used to prevent late blight (Fry 2016; Small et al. 2015); however, new *P. infestans* strains have emerged, with some resistant to the fungicides (Hu et al. 2012; Kirk et al. 2001). Consequently, late blight is increasingly becoming more difficult to control.

Breeding new varieties with genetic resistance to *P. infestans* is the most effective way to minimize late-blight-caused losses. Thus, the effective use of new resistance (*R*) genes by breeders is essential. The resistance to *P. infestans* is mediated by *R* genes and several quantitative trait loci (QTLs). Over several decades, researchers have identified late blight *R* genes, including *Ph-1*, *Ph-2*, *Ph-3*, *Ph-4*, and *Ph-5*, in wild tomato species. The *Ph-1* gene, which confers complete resistance to *P. infestans* race T₀, was mapped to the end of chromosome 7 (Bonde and Murphy 1952; Peirce 1971). The *Ph-2* gene, which was identified in *S. pimpinellifolium* ‘West Virginia 700’ (Gallegly and Marvel 1955), confers partial late blight resistance, which is useful for slowing the spread of the disease (Black et al. 1996a; Goodwin et al. 1995). The *Ph-2* gene was mapped to an 8.4-centimorgan (cM) region between markers CP105 and TG233 on chromosome 10 (Moreau et al. 1998). The *Ph-3* gene was identified in *S. pimpinellifolium* ‘LA3708’ (AVRDC 1994) and is a partially dominant gene located on the long arm of chromosome 9, where it encodes a coiled-coil nucleotide binding site leucine-rich repeat (CC-NBS-LRR) *R* protein (Black et al. 1996a,b; Chunwongse et al. 2002; Zhang et al. 2013, 2014). The *Ph-4* gene was detected in *S. habrochaites* ‘LA1033’ and was reported as a potential QTL (AVRDC 1998; Lough 2003). The *Ph-5* gene was identified in *S. pimpinellifolium* accession PI 270443, which is resistant to at least seven late blight pathogen races (Foolad et al. 2006). However, *Ph-5* is controlled by two sites, one on chromosome 1 (named *Ph-5-1*) and one on chromosome 10 (named *Ph-5-2*) (Merk and Foolad 2012; Merk et al. 2012; Nowicki et al. 2012), which has increased the difficulty of its application. Additionally, some late blight resistance QTLs have been identified in *S. habrochaites* (Brouwer et al. 2004; Chen et al. 2008; Li et al. 2011), *S. cheesmaniae* (Lukyanenko 1991), and *S. pennellii* (Smart et al. 2007). Unfortunately, introgressing these disease resistance QTLs into cultivated tomato plants requires more time, and many of them may be associated with undesirable traits (Brouwer et al. 2004). To date, the QTLs conferring late blight resistance have not been successfully applied to commercial production, and their practical value remains unknown (Foolad et al. 2014).

A previous study revealed that combining *Ph-2* and *Ph-3* results in resistance to the major *P. infestans* races in tomato (Foolad et al. 2006). Therefore, combining these two genes via molecular marker-assisted selection is an effective breeding strategy for improving tomato late blight resistance. Although

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Ph-3 has been cloned, the lack of detailed information regarding *Ph-2*, including its genomic location, has limited the development of molecular markers useful for the efficient selection of this gene. The objectives of this study were to (i) screen for

markers linked to *Ph-2* and construct a high-resolution linkage map using the markers, (ii) analyze candidate *Ph-2* genes, and (iii) assess the accuracy and applicability of the developed markers using other tomato lines.

Table 1. Details regarding the markers associated with *Ph-2* on chromosome 10 of tomato and used in this study

Marker name ^y	Primer sequence (5'–3') ^z	Position in chromosome 10 (SL2.50)	Enzyme
InDel-1	F: CCTTTTCGAGAGTCAATCTG R: TGATGTGAGGAAGTGCTTTA	63,865,736–63,865,755 63,865,852–63,865,833	...
InDel-2	F: CAGTACCTAACCACCATTTGAA R: GCCGTTATTGAGGTTTATTG	64,488,568–64,488,588 64,488,839–64,488,820	...
InDel-3	F: TTTGCACTCTATGATCACCA R: AACGGTGCTTGAAGTGTATT	64,511,947–64,511,966 64,512,173–64,512,154	...
InDel-4	F: TTGCTAACGAATTCACCTCT R: ATCAAACCTAGCTATTCCA	64,539,696–64,539,715 64,539,883–64,539,864	...
InDel-a	F: TAAAGGGATGGTTTGTGGGGG R: GGGTCCTTGTCTGAACCTAGAT	64,543,045–64,543,065 64,543,140–64,543,117	<i>DdeI</i>
CAPS-e	F: ATTGCCAAATCTGGTACTCTGGT R: CGGATAACAACCTGACAGCTCTA	64,552,836–64,552,858 64,553,071–64,553,049	<i>BfaI</i>
SNP-1	F: TCCAGATTCCCTCAGCATTCTTC R: CGGATAACAACCTGACAGCTCTA	64,552,871–64,552,893 64,553,071–64,553,049	...
KASP-1	FAM: CAATTCCTACTTTGCACCAGCA HEX: ACAATTCCTACTTTGCACCAGCT Common: TGTGCTTAATTCTA TAAATGACAAAACT	64,552,952–64,552,972 64,552,951–64,552,973 64,553,025–64,552,997	...
InDel-b	F: TTCATGTACGTTGTACAATAATCT R: ATTGCATCTAGAACTCCGGTGTG	64,556,588–64,556,612 64,556,827–64,556,805	...
InDel-c-1	F: TCATCCGTCAGAAATAACCCCA R: TGTAATGCCACACAAGCAGA	64,570,133–64,570,154 64,570,310–64,570,291	...
InDel-c	F: AAAAAATTATCACTAAGAAGTTTGGT R: GTAATGCCACACAAGCAGAA	64,570,206–64,570,230 64,570,309–64,570,290	...
InDel-d	F: ACGTATCGCAGAAAGTTGTTGTG R: TTCTAGTGGCACAACAGCTGTTA	64,573,682–64,573,704 64,573,956–64,573,934	...
CAPS-a	F: TAGGGAGATAGGCGAGCAGG R: TTTGGTGAGTCCATGTGAGC	64,575,341–64,575,360 64,575,566–64,575,547	...
InDel-e	F: AATGCAACGGCTAATCAACACA R: AAGCAAATGATTACCCGAGA	64,576,952–64,576,973 64,577,021–64,577,001	<i>BfaI</i>
InDel-f	F: ACATATAGCGATAATTGAGGTAGA R: CATGCTATCATGTGCCTCTGTAAT	64,580,960–64,580,984 64,581,230–64,581,207	...
InDel-g	F: TGACTTACCCTCCTCTTATTAATAC R: AGAGGAAAGTGAACTAGGAAGCT	64,581,949–64,581,973 64,582,248–64,582,225	...
InDel-h	F: TTTGGTTGCTTGAATATTTCTT R: AGTGCCTTTTAACTTCAATT	64,585,267–64,585,289 64,585,528–64,585,506	...
CAPS-b-1	F: GGCCGGTAGGAGCAACATT R: TTTGCTTAGCTCGATAGCA	64,603,850–64,603,868 64,603,934–64,603,915	<i>Tsp509I</i>
CAPS-b-2	F: GGCCGGTAGGAGCAACATT R: AGTTGAATTTTCGCTTAGCTCGAT	64,603,850–64,603,869 64,603,941–64,603,919	<i>DdeI</i>
SNP-6	F: GGCCGGTAGGAGCAACATT R: AGTTGAATTTTCGCTTAGCTCGAT	64,603,850–64,603,869 64,603,941–64,603,919	...
SNP-2	F: TATTTCTGTACAGTTGGGAGAGG R: AAGGATCCTGCATATTGTTGGCT	64,626,301–64,626,323 64,626,533–64,626,511	...
CC-Ase	R: GCTGACCGACAAATTCCTTTA F: TTATTGAGTGGAAGGGAGAT	64,627,112–64,627,131 64,627,595–64,627,614	<i>AseI</i>
InDel-i	F: GCCCAAACAAGAGAGGAACG R: GCGATTCAATCATAACGGTGTCT	64,633,429–64,633,448 64,633,716–64,633,695	...
CAPS-d	F: TCAGAACATCCTAAAGACCGT R: GGTATGTGGGTTGCGGATCA	64,633,681–64,633,702 64,633,988–64,633,969	<i>Hpy99I</i>
InDel-j	F: AGCACCATTATGATTGAATCGC R: TCATGCCGATCAGCTTACCC	64,633,695–64,633,716 64,633,971–64,633,952	...
SNP-3-2	F: AAGCACCATTATGATTGAATCGC R: TGCCGATCAGCTTACCCTTTTTA	64,633,694–64,633,716 64,633,968–64,633,946	...
SNP-3-1	F: CACCGTTATGATTGAATCGACA R: TGCCGATCAGCTTACCCTTTTTA	64,633,697–64,633,719 64,633,968–64,633,946	...
CAPS-c	F: AGCACCATTATGATTGAATCGC R: TCATGCCGATCAGCTTACCC	64,633,695–64,633,716 64,633,971–64,633,952	<i>PsiI</i>

(Continued on next page)

^y InDel = insertion and deletion, CAPS = cleaved amplified polymorphic sequence, SNP = single-nucleotide polymorphism, and KASP = Kompetitive allele-specific PCR.

^z F = forward primer and R = reverse primer.

Materials and Methods

Plant materials. The resistant line *S. lycopersicum* ‘Moboline’ (accession number LA3152) containing the *Ph-2* gene (kindly provided by the Tomato Genetics Resource Center [TGRC] at the University of California, Davis, CA, U.S.A.) and the susceptible line *S. lycopersicum* LA3988 (an introgression line of *S. habrochaites* ‘LA1777’ and *S. lycopersicum* ‘FM-6203’, kindly provided by TGRC) were selected as parents. The F₂ segregating population obtained from the Moboline and LA3988 hybridization contained 394 individuals and was used for fine mapping the *Ph-2* gene.

In all, 35 inbred lines were used to verify the accuracy of the molecular markers linked to *Ph-2* (Supplementary Table S1). Thirteen of these were kindly provided by TGRC, one of which contains *Ph-2* (<https://tgrc.ucdavis.edu/>). Six of the inbred lines were kindly provided by the Mountain Horticultural Crops Research and Extension Center (Mills River, NC, U.S.A.), including one carrying *Ph-2* (<https://mountainhort.ces.ncsu.edu/>). Five of the inbred lines were kindly provided by the Asian Vegetables Research and Development Center (Taiwan, China), one of which contains *Ph-2* (<https://avrdc.org/>). The remaining 11 inbred lines were kindly provided by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences.

DNA extraction and marker development. For both the parents and progenies, fresh leaves were collected from individual plants. Total genomic DNA was extracted according to the cetyltrimethylammonium bromide method (Fulton et al. 1995). The DNA concentration was adjusted to 40 ng/μl for a PCR.

In a previous study, *Ph-2* was localized to chromosome 10 between markers CP105 and TG233 (Moreau et al. 1998). The Primer 5.0 software (<http://www.PremierBiosoft.com>) was used to design cleaved amplified polymorphic sequence (CAPS) and insertion and deletion (InDel) markers targeting this interval based on the publicly available tomato genome resequencing data (<https://solgenomics.net/>). The primers were 14 to 28 bp long, with annealing temperatures of

55 to 60°C. The PCR products for the CAPS and InDel markers were 80 to 500 and 80 to 300 bp long, respectively. All primers were synthesized by Sangon Biotech Co., Ltd. (Beijing, China).

To delimit the *Ph-2* candidate region, markers were developed based on the whole-genome resequencing data (30× genome coverage) for *S. lycopersicum* ‘Moboline’ (resistant material) and the near-isogenic line *S. lycopersicum* ‘Monalbo’ (susceptible material; accession number LA2818, kindly provided by TGRC). The genomes were sequenced by Anoroda Gene Technology Co., Ltd. (Beijing, China). The integrity and purity of DNA samples were evaluated by 1% agarose gel electrophoresis, whereas the DNA concentration was determined with the Qubit 2.0 Fluorometer (Life Technologies Corporation, Carlsbad, CA, U.S.A.). The DNA samples were randomly broken into 350-bp fragments with the Covaris M220 ultrasonicator (Gene Company Limited, Beijing, China). Sequencing libraries were constructed with the TruSeq Library Construction Kit (Illumina, San Diego, CA, U.S.A.). After the cluster generation step, the libraries were sequenced with the Illumina HiSeq 2000 system, resulting in 150-bp paired-end reads. We obtained clean reads by removing the adapter reads as well as reads with >10% unknown nucleotides and low-quality reads (i.e., reads with >50% bases with a Q value ≤ 5) from the raw data with FastQC (Andrews 2010) and Cutadapt (Martin 2011). The clean reads were mapped to the tomato Heinz 1706 reference genome (SL2.50 version) with the SOAP software. The SAMtools program (version 1.2) (Li et al. 2009) was used to sort reads, whereas Picard tools (version 1.13) (<http://broadinstitute.github.io/picard/>) were applied to eliminate duplicate reads due to PCR. Subsequently, the Genome Analysis Toolkit (McKenna et al. 2010) and the HaplotypeCaller protocol were used for calling single-nucleotide polymorphisms (SNPs) and InDels via the local reassembly of haplotypes for populations. The insertion and deletion mutations were used to design InDel markers. Regarding the SNPs, the Lasergene software (DNASTar, Madison, WI, U.S.A.) was used to scan for specific restriction enzyme recognition sites to develop CAPS markers.

Table 1. (Continued from previous page)

Marker name ^y	Primer sequence (5′–3′) ^z	Position in chromosome 10 (SL2.50)	Enzyme
SNP-4-2	F: AAGCACCGTTATGATTGAATCGC R: TGCCGATCAGCTTACCCCTTTTA	64,633,694–64,633,716 64,633,968–64,633,946	...
SNP-4-1	F: CACCGTTATGATTGAATCGCACA R: TGCCGATCAGCTTACCCCTTTTA	64,633,697–64,633,719 64,633,968–64,633,946	...
SNP-5	F: AGTCGAAATTCAAACATGTTCTCT R: TCCCAAAGCCTATCTATTACGCA	64,641,874–64,641,897 64,642,147–64,642,124	...
InDel-k-1	F: CGCCAACACCAACAACCAAA R: CGTGTGGGTCAAACACATGG	64,768,173–64,768,192 64,768,292–64,768,272	...
InDel-k-2	F: CATCAACTCCTAGTTTCTTCTGAG R: CGTGTGGGTCAAACACATGG	64,768,040–64,768,064 64,768,292–64,768,272	...
CAPS-1	F: GTCAATAATTTTCAACCATAGAATGATT R: TGACATGAGAAGGAAAAGACTTAAG	64,768,441–64,768,465 64,768,745–64,768,721	HinfI
InDel-A	F: TAGTGGAGGTAGGATTAT R: AGTAAGACTAAAAGTGGAA	64,877,340–64,877,357 64,877,545–64,877,527	...
InDel-B	F: ATTCAGTAAAGGGGACA R: TCGTTATTGGAGGATGG	64,913,905–64,913,923 64,914,023–64,914,007	...
InDel-5	F: CCGAGATGAGAGATTATACCA R: CGTTAGGAAATCTTGTTCG	64,944,482–64,944,502 64,944,761–64,944,742	...
InDel-C	F: CTTCCACGATTACACCT R: TAATTCGTGACACCATA	64,959,501–64,959,517 64,959,625–64,959,609	...
InDel-6	F: AGAGAAAGAAACAAATCCCC R: CTACAACATTGCACAGCTA	65,310,385–65,310,404 65,310,570–65,310,551	...
InDel-D	F: CTTACCTTCTTCTCCAA R: CTTATTACCTTTACCAAA	65,346,520–65,346,536 65,346,749–65,346,732	...
InDel-F	F: TTGGATTATGTTGGGTAG R: CAGTTCCTTAGGCTTT	65,390,746–65,390,763 65,390,967–65,390,951	...
InDel-E	F: CTCCAAACAAGGAAAGA R: ATGCCAAAGGGCTAAAA	65,411,709–65,411,725 65,411,929–65,411,913	...

The remaining polymorphic SNPs were developed as Kompetitive allele-specific PCR markers. Details regarding the primers for the identified markers are listed in Table 1.

Inoculation and disease assay. The *P. infestans* race T₁ isolate used as the inoculum can overcome the resistance mediated by *Ph-1* but not the resistance due to *Ph-2*. The isolate was stored in 15% dimethyl sulfoxide at -80°C (Zhang et al. 2013). Before inoculating plants, the isolate was activated and propagated on the leaflets of susceptible line LA3988, as described by Foolad et al. (2014). Plants with five fully expanded leaves were then spray inoculated with a suspension comprising 2×10^4 sporangia/ml, after which they were incubated at $20 \pm 2^\circ\text{C}$ and 100% relative humidity in darkness. After 24 h, the plants were maintained at $20 \pm 2^\circ\text{C}$ and 70 to 90% relative humidity with a 12-h photoperiod in a greenhouse (natural light intensity) for 7 days (Brouwer et al. 2004; Zhang et al. 2013).

Disease severity (DS) was rated 7 to 10 days after the inoculation using the following 0-to-6 scale: 0 = no apparent symptoms; 1 = 1 to 5% of the leaf area affected, with small lesions; 2 = 6 to 15% affected, with restricted lesions; 3 = 16 to 30% affected, with water-soaked flecks on the stem; 4 = 31 to 60% affected, with a few stem lesions; 5 = 61 to 90% affected, with expanding stem lesions; and 6 = 91 to 100% affected, with a severely damaged stem or dead plants. Plants with a DS score of 0 to 4 were considered resistant, whereas those with a DS score of 5 to 6 were designated as susceptible (Zhang et al. 2013, 2014).

Genetic mapping. In total, 394 individuals of the F₂ segregating population were genotyped with 11 molecular markers to map the *Ph-2* gene. Polymorphic marker bands were distinguished as follows: maternal genotype (genotype a), paternal genotype (genotype b), heterozygous (genotype h), or missing data (genotype u). A genetic linkage map was constructed using JoinMap 4 (Van Ooijen 2006), with a minimum logarithm of odds threshold of 3.0. Specifically, the map order and genetic distances were estimated based on the maximum likelihood and Kosambi function (Kosambi 1943).

Candidate gene analysis. The number and annotation details of the genes in the *Ph-2* candidate interval were obtained from the Sol Genomics Network (https://solgenomics.net/jbrowse_solgenomics/?data=data%2Fjson%2FSL2.50&loc=SL2.50ch10%3A64570206..64768173&tracks=DNA%2Cgene_models&highlight=).

Sequence alignment and prediction of the structure of the proteins encoded by candidate genes. The coding regions of the candidate genes differentially expressed between the resistant and susceptible materials were sequenced and aligned. The coding region of each candidate gene based on the Heinz 1706 genome was downloaded from the Sol Genomics Network (<https://solgenomics.net/search/locus>). Sequencing primers were designed with the Primer 5.0 software (<http://www.PremierBiosoft.com>). The PCR products were 500 to 1,000 bp and the adjacent amplified fragments overlapped by at least 90 bp. PCR products were sequenced by Sangon Biotech Co., Ltd. Sequences were aligned with MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>). Moreover, protein structures were predicted with the Conserved Domains program (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?>).

Statistical analysis. Data were analyzed with Microsoft Excel. Additionally, the χ^2 test was performed with SPSS 17.0 software (SPSS Inc, Chicago, IL, U.S.A.).

Results

Inheritance analysis of late blight resistance mediated by *Ph-2*.

In earlier investigations, *Ph-2* was described as a single, incompletely dominant gene for late blight resistance (Gallegly and Marvel 1955; Moreau et al. 1998). In this study, we assessed the severity of late blight in the parental lines as well as in the F₁ and F₂ populations. The resistant line Moboline either lacked lesions or had narrow restricted lesions (Fig. 1A), with a DS score of 1.25 (DS scores between 0 and 2, standard deviation = 0.55, $n = 20$) (Fig. 1B). The susceptible LA3988 plants had extensive expanded lesions or were dead (Fig.

Fig. 1. Phenotypes of Moboline, F₁, and LA3988 plants 8 days after being inoculated with *Phytophthora infestans* and disease assays of the parental lines and the F₂ population. **A**, Phenotypes of Moboline, F₁, and LA3988 plants inoculated with *P. infestans*. **B**, Mean disease severity (DS) scores among Moboline, LA3988, and F₁ plants. **C**, Mean DS scores for a, h, and b plants genotyped by the closest marker. **D**, Frequency distribution of the DS scores in the F₂ population. Asterisks (**) indicate $P < 0.01$ based on Duncan's multiple range test.

1A), with a DS score of 5.25 (DS scores between 5 and 6, standard deviation = 0.44, $n = 20$) (Fig. 1B). These results confirmed that Moboline is significantly more resistant to late blight than LA3988 ($P < 0.05$). The F_1 plants had an intermediate DS score of 2.75 (DS scores between 2 and 4, standard deviation = 0.72, $n = 20$)

(Fig. 1B), which was significantly different from the DS scores of either parent and the midparental value (DS score = 3.25, standard deviation = 0.30, $n = 20$) ($P < 0.05$). These values were indicative of the incomplete dominance of *Ph-2*. Analyses of the DS and genotype of 394 individuals of the F_2 population revealed that 88, 202, and 104

Fig. 2. Genetic and physical maps of *Ph-2*. **A**, Genetic map of the *Ph-2* locus. Indel = insertion and deletion, KASP = Kompetitive allele-specific PCR, and CAPS = cleaved amplified polymorphic sequence. **B**, Physical fine mapping of *Ph-2*. **C**, Five resistance genes in the *Ph-2* candidate region.

Table 2. Genotypes and phenotypes of recombinant individuals^z

Generation	Code	Genotype				Phenotype
		InDel-4	KASP-1	CC-Ase	CAPS-1	
F_2 recombinant individual	A162	h	h	b	b	S
	A236	b	b	b	h	S
	A283	h	h	h	b	R
	A343	h	h	h	b	S
F_3 recombinant individual	A343-23	a	a	a	b	S
	A283-8	a	a	a	b	R
	A162-14	a	a	b	b	S

^z InDel = insertion and deletion, KASP = Kompetitive allele-specific PCR, CAPS = cleaved amplified polymorphic sequence, a = homozygous for the resistant parent Moboline, b = homozygous for the susceptible parent LA3988, h = heterozygous, S = susceptible, and R = resistant.

individuals carried the a, h, and b genotypes, respectively. The mean DS scores for the a, h, and b genotypes were 2.59, 3.43, and 5.06, respectively. There were significant differences among these three genotypes ($P < 0.05$) (Fig. 1C). The χ^2 test indicated that the segregation ratio of the a, h, and b progenies was consistent with the expected ratio of 1:2:1 ($\chi^2 1:2:1 = 1.553$, $P = 0.460$). These results confirmed the incomplete dominance of the *Ph-2* gene.

Fine mapping of the *Ph-2* gene. In a previous study, *Ph-2* was mapped within an 8.4-cM region between markers CP105 and TG233 on chromosome 10 (Moreau et al. 1998). On the basis of the results of previous studies, 18 molecular markers were screened in a larger interval, 7 of which are polymorphic between the parental lines Moboline and LA3988 (Table 1). A linkage analysis of 394 *F*₂ individuals identified InDel-4 and CAPS-1 as the nearest markers flanking the *Ph-2* locus, with an interval of 229 kb in the tomato Heinz 1706 reference genome (SL2.50 version) (Fig. 2A).

To narrow down the *Ph-2* candidate region, near-isogenic lines of Moboline (resistant line) and Monalbo (susceptible line) were sequenced (30x effective depth). In total, 54 SNPs and 60 InDels were detected in the *Ph-2* candidate region (Supplementary Table S2). On the basis of these polymorphisms, 35 new markers were designed, 4 of which were polymorphic between the parents of the fine-mapping population (Table 1). These four polymorphic markers were used to further delimit the fine mapped interval to a 141-kb region (Fig. 2B) in the reference genome between the CAPS-1 and CC-Ase markers (Fig. 2A). This result was confirmed by the phenotypes of the *F*₃ progenies corresponding to three of the most informative *F*₂ recombinants (Table 2).

***Ph-2* candidate gene analysis.** The annotation of the *Ph-2* candidate region based on the fine-mapping data revealed 21 genes (Supplementary Table S3), including 5 putative *R* genes (Fig. 2C), with 1 belonging to the NBS-LRR family (*Solyc10g085460*) and 4 encoding

serine/threonine kinases (*Solyc10g085570*, *Solyc10g085490*, *Solyc10g085600*, and *Solyc10g085660*) (Table 3).

An alignment of the coding regions of the five projected *R* genes indicated that only *Solyc10g085460* varied between the parents (Supplementary File S1). The coding sequence of this putative gene comprises 3,687 bp, with 32 SNPs between Moboline and LA3988. Additionally, *Solyc10g085460* was predicted to encode a CC-NBS-LRR protein consisting of 1,228 amino acids, of which 19 differ between Moboline and LA3988. The predicted protein structure suggested that these 19 amino acids are located in the LRR conserved domain and are associated with the diversity in the number of LRRs in Moboline (17 LRRs) and LA3988 (16 LRRs) (Fig. 3). Accordingly, *Solyc10g085460* is presumed to play a key role in the *Ph-2*-mediated late blight resistance of tomato.

Verification of the accuracy of the molecular markers linked to *Ph-2*. The molecular markers linked to *Ph-2* (namely, InDel-1, InDel-2, InDel-3, InDel-4, InDel-c, CC-Ase, CAPS-1, and InDel-6) were used to screen 35 tomato inbred lines (Supplementary Table S1). The InDel-4, InDel-c, and CAPS-1 markers correctly determined the *Ph-2* genotype of all 35 lines, with 100% accuracy (Fig. 4). In contrast, the InDel-1, InDel-2, InDel-3, CC-Ase, and InDel-6 markers were 91.43, 51.43, 40, 74.29, and 94.29% accurate, respectively.

Discussion

Breeding resistant varieties is the most effective way to prevent late blight. Currently, *Ph-2* and *Ph-3* are the most widely used *R* genes in commercial tomato cultivars. Newly emerging and more aggressive *P. infestans* strains are able to infect tomato plants carrying only one of these genes (Chunwongse et al. 2002; Foolad et al. 2008; Irzhansky and Cohen 2006). Researchers have confirmed that combining *Ph-2* and *Ph-3* can enhance the resistance to multiple races

Table 3. Annotated *Ph-2* candidate genes

Gene name	Start	End	Annotation ^z
<i>Solyc10g085460</i> (–)	64,626,257	64,630,102	CC-NBS-LRR resistance protein
<i>Solyc10g085570</i> (+)	64,684,109	64,697,016	CTR4 and its splice variants CTR4sv1 and -sv2
<i>Solyc10g085490</i> (–)	64,643,337	64,646,939	F-box protein PP2-B1
<i>Solyc10g085600</i> (–)	64,715,492	64,728,193	F-box/LRR-repeat protein 4
<i>Solyc10g085660</i> (–)	64,768,049	64,769,422	F-box protein SKIP24

^z CC-NBS-LRR = coiled-coil nucleotide binding site leucine-rich repeat.

Fig. 3. Predicted structure of the protein encoded by *Solyc10g085460* in Moboline and LA3988. NB-ARC = nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 and LRR = leucine-rich repeat.

Fig. 4. Gel electrophoresis of molecular markers linked to *Ph-2* in 35 tomato inbred lines. M = size marker; varieties as follows: lanes 1 to 5 = N735, N736, 17-3051, NC25P, and 17-3052; lanes 6 to 10 = NC2CECBR, 18-S88-1, NEBR-7, 18-S105-1, and NCEBR-8; lanes 11 to 15 = 18-S120-1, 18-S87-1, CLN3125A, 16-350-1, and CLN3078B; lanes 16 to 20 = 14-1772, CLN3241R, 18-S122-1, CLN2037E, and 18-S143-1; lanes 21 to 25 = CLN2037B, 18-S71-1, Monalbo, E6203, and Moneymaker; lanes 26 to 30 = M82, VFN8, H1706, Pearson, and CASTLEMART; lanes 31 to 35 = Momotaro, Ailsa Craig, Micro-Tom, KR1441, and Moboline; S contains *ph-2* and R contains *Ph-2*.

(Gardner and Panthee 2010; Panthee and Gardner 2010). Pyramiding multiple *R* genes to develop highly resistant varieties represents an effective strategy for minimizing late-blight-caused losses. The *Ph-3* gene has been cloned (Zhang et al. 2014) and the resistance conferred by this gene can be efficiently selected via marker-assisted selection (Robbins et al. 2010). However, *Ph-2* has not been cloned and its precise genomic position was unknown, making it challenging for researchers attempting to develop efficient molecular markers to improve selection efforts. To increase the utility of *Ph-2*, we studied its inheritance and developed closely linked molecular markers.

A previous study revealed that *Ph-2* is located on the distal part of the long arm of chromosome 10 (Moreau et al. 1998). In this study, we used an F₂ population developed from a cross between the late-blight-resistant line Moboline and the susceptible line LA3988 to fine map *Ph-2*. Whole-genome resequencing facilitated the development of molecular markers saturating the *Ph-2* interval. We constructed a high-resolution genetic map with 11 polymorphic molecular markers. Moreover, *Ph-2* was localized to a 141-kb interval (64,627,614 to 64,768,745 bp) between markers CC-Ase and CAPS-1 on chromosome 10, corresponding to a genetic distance of 0.27 cM.

In plants, the largest *R* gene family encodes NBS and LRR domains. Furthermore, 26 late-blight *R* genes have been cloned in tomato and potato, 22 of which belong to the NBS-LRR *R* gene family (Rodewald and Trognitz 2013; Xu and Jin 2017; Yue et al. 2012; Zhang et al. 2013). In this study, we detected 21 genes in the *Ph-2* candidate region but only *Solyc10g085460* belongs to the NBS-LRR gene family. A sequence analysis revealed differences in the *Solyc10g085460* coding sequence between the resistant and susceptible materials, resulting in diverse protein structures. Therefore, our findings imply that *Solyc10g085460* may be the *Ph-2* gene or is possibly involved in the same pathway as *Ph-2* during responses to *P. infestans*.

In this study, the accuracy of the markers CC-Ase and InDel-c, which are closest to *Ph-2*, were 74.29 and 100.00%, respectively. Consequently, InDel-c may be useful for tomato breeders interested in developing new varieties carrying *Ph-2*. However, very few resistant cultivars were tested using the newly developed markers in this study. Therefore, it may be necessary to verify the generality and the accuracy of the markers in a larger population in a future study.

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