The Ph-3 gene from *Solanum pimpinellifolium* encodes CC-NBS-LRR protein conferring resistance to *Phytophthora infestans*

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Abstract

**Key message** Ph-3 is the first cloned tomato gene for resistance to late blight and encodes a CC-NBS-LRR protein.

Abstract Late blight, caused by *Phytophthora infestans*, is one of the most destructive diseases in tomato. The resistance (*R*) gene Ph-3, derived from *Solanum pimpinellifolium* L3708, provides resistance to multiple *P. infestans* isolates and has been widely used in tomato breeding programmes. In our previous study, Ph-3 was mapped into a region harbouring *R* gene analogues (RGA) at the distal part of long arm of chromosome 9. To further narrow down the Ph-3 interval, more recombinants were identified using the flanking markers G2-4 and M8-2, which defined the Ph-3 gene to a 26 kb region according to the Heinz1706 reference genome. To clone the Ph-3 gene, a bacterial artificial chromosome (BAC) library was constructed using L3708 and one BAC clone B25E21 containing the Ph-3 region was identified. The sequence of the BAC clone B25E21 showed that only one rGA was present in the target region. A subsequent complementation analysis demonstrated that this rGA, encoding a CC-NBS-LRR protein, was able to complement the susceptible phenotype in cultivar Monneymaker. Thus this RGA was considered the Ph-3 gene. The predicted Ph-3 protein shares high amino acid identity with the chromosome-9-derived potato resistance proteins against *P. infestans* (Rpi proteins).

Introduction

Late blight, caused by *Phytophthora infestans*, is one of the most devastating diseases for field-grown tomatoes. Under favourable conditions, *P. infestans* can spread at an alarming pace, and the compatible host will be devastated within 7–10 days (Fry 2008). Fungicide treatment is currently the most common method to control late blight. However, fungicide application is costly and has a negative impact on human health and environmental safety. Moreover, the pathogen quickly evolves and some of the new variants are insensitive to commonly used fungicides (Goodwin et al. 1996). The disease is especially problematic for organic growers who do not use any chemical pesticides in the production process. Therefore, introduction of resistances from wild tomato species into cultivated tomato is considered as a valuable method to achieve durable late blight resistance.
Currently, more than 60 *Solanum* resistance genes against *P. infestans* (*Rpi* genes), mainly in potato, have been located in 16 regions on 10 chromosomes (Rodewald and Trognitz 2013). Among them, some have been cloned through map-based cloning or allele mining (rodewald and Trognitz 2013). Most of the *Rpi* genes are identified in wild potatoes, such as *S. demissum*, *S. bulbocastanum*, *S. venturii*, etc. (Vleeshouwers et al. 2011; Rodewald and Trognitz 2013). In tomato, much less studies on late blight resistance have been carried out. This is in part because this pathogen in tomato was not as prevalent as in potato, at least before the 1990s when many of the potato isolates were not pathogenic to tomato (Nowicki et al. 2012). However, tomato *P. infestans* isolates have recently undergone significant genetic changes and are becoming one of the most devastating pathogens for tomato cultivation (Foolad et al. 2008).

*P. infestans* is heterothallic, and both A1 and A2 mating types are required for completion of the sexual cycle. Sexual reproduction results in high levels of genetic variation in the offspring and may lead to rapid pathogen evolution and thus increases the risk of epidemics (Foolad et al. 2008). In the latest reports, tomato *P. infestans* isolates collected in China and Tunisia are still A1 mating types (Guo et al. 2010; Li et al. 2013; Harbaoui et al. 2013). In the USA, however, the predominant clonal lineage US-22 in Wisconsin is A2 mating type and resulted in the epidemics on tomato in 2009 (Gevens and Seidl 2013). The A2 mating type of tomato *P. infestans* isolates has also been reported in Russia (Statsyuk et al. 2010). In South-West India, appearance of the (blue) 13_A2 lineage caused severe outbreaks of late blight on tomatoes from 2009 to 2010 (Chowdappa et al. 2013). In potato cultivation, the aggressive 13_A2 lineage has emerged in Northwest Europe and rapidly replaced other genotypes (Cooke et al. 2012). This lineage is also present in the population of potato *P. infestans* in China (Li et al. 2013), but has not been collected yet in the Chinese tomato *P. infestans* population.

Due to the recent increased significance of tomato late blight, more effort is needed to identify genetic resources for late blight resistance and transfer the resistance to breeding lines and cultivars. To date, resistance to *P. infestans* has been reported in wild tomato species. The *Ph-1* gene is the first reported *Rpi* gene in tomato, which is a dominant gene mapped on chromosome 7 and provides resistance against *P. infestans* isolate T_0_. The *Ph-1* gene was originally identified in *Solanum pimpinellifolium* accessions known as West Virginia 19 and 731 and has been introduced into the cultivated tomato (Bonde and Murphy 1952; Gallegly and Marvel 1955; Rich et al. 1962; Peirce 1971). The second *Rpi* gene *Ph-2* was identified in another *S. pimpinellifolium* accession (West Virginia 700) (Gallegly and Marvel 1955). The *Ph-2* gene, conferring incomplete late blight resistance, was mapped into an 8.4-cM interval on the long arm of chromosome 10 (Moreau et al. 1998). This gene provides partial resistance resulting in only a reduction in the rate of disease development (Goodwin et al. 1995; Black et al. 1996a). Resistance conferred by both *Ph-1* and *Ph-2* was overcome by different *P. infestans* isolates from China, Indonesia, Nepal and the Philippines (AVRDC 1995, 1998, 1999), which prompted further screening of tomato germplasm for new *Rpi* genes. As a result, *S. pimpinellifolium* L3708 was found to be highly resistant to a wide range of *P. infestans* isolates overcoming *Ph-1* and *Ph-2* (Black et al. 1996a, b). The late blight resistance in L3708 is conditioned by a partially dominant gene, *Ph-3*, which was mapped on the long arm of chromosome 9 (Black et al. 1996a; Chunwongse et al. 2002; Zhang et al. 2013). With marker-assisted selection (MAS) using *Ph-3*-linked molecular markers, this gene has been successfully introgressed into tomato breeding lines and tomato cultivars for both commercial processing and fresh-market (Foolad et al. 2008; Gardner and Panthee 2010a, b; Panthee and Gardner 2010; Robbins et al. 2010). However, the resistance conferred by *Ph-3* is also race-specific, and the isolates virulent on L3708 have already been identified (Chunwongse et al. 2002). Another reported late blight resistant accession is *S. habrochaites* LA1033, which was designated as the source of *Ph-4* (AVRDC 1998). LA1033 was used as one of the differential hosts to classify tomato *P. infestans* isolates (Kim and Mutschler 2000; Chunwongse et al. 2002). Characterization of *Ph-4* has been hampered because follow-up investigations revealed that the resistance in LA1033 was actually controlled by multiple quantitative trait loci (QTLs) (Lough 2003; Kim and Mutschler 2000). Recently, a new resistant line, *S. pimpinellifolium* PSLP153, has been discovered which showed resistance against seven different *P. infestans* isolates (Foolad et al. 2006, 2008). Two genomic regions on chromosome 1 (tentatively named *Ph-5-1*) and chromosome 10 (tentatively named *Ph-5-2*) were identified through a selective genotyping approach (Merk et al. 2012; Merk and Foolad 2012; Nowicki et al. 2012). Efforts are underway to develop commercial breeding lines and hybrid cultivars containing these resistance genes in combination with *Ph-2* and *Ph-3* (Foolad et al. 2008; Nowicki et al. 2012).

Other QTLs conferring race-non-specific resistance have been identified from *S. pennellii* and *S. habrochaites* (Smart et al. 2007; Brouwer et al. 2004; Brouwer and St. Clair 2004; Li et al. 2011a). However, the effects of these QTLs are relatively small and prone to environmental influences. Moreover, linkage drag might complicate the use
of these QTls in breeding programmes (Brouwer and St. Clair 2004).

Currently, introgression or pyramiding of R genes via traditional breeding may not always be possible or too time-consuming. An alternative approach to introduce single or multiple R genes is genetic transformation (Halpin 2005). To achieve durable resistance, three potato Rpi genes were introduced into one genotype through a one-step transformation strategy, and the resulting plants showed an expected broadened resistance spectrum (Zhu et al. 2012, 2013). This approach, however, requires prior knowledge of the gene(s), including mapping, cloning, and functional characterization.

In this study, we performed map-based cloning to isolate Ph-3, a gene encoding a coiled-coil nucleotide-binding leucine-rich repeat (CC-NBS-LRR) protein. Further, we analysed the Ph-3 protein structure and compared it with other Rpi proteins characterized so far from potato.

### Materials and methods

#### Plant materials

The *S. lycopersicum* accessions CLN2037B and CLN2037E, containing the Rpi gene Ph-3, were kindly provided by the Asian Vegetable Research and Development Center (AVRDC). These two cultivars were crossed with the susceptible tomato breeding line 02393, respectively. Recombinant screening was conducted using the F2 seeds with Ph-3 flanking markers G2-4 and M8-2 (Table 1). In addition, eight F2 families (B212, B481, N299, N337, N1036, N1097, N1200, N1384), which were derived from the cross between CLN2037B and LA4084 (susceptible) and identified in our previous study (Zhang et al. 2013), were also used for screening recombinants (about 150 plants per F2 family) with the same markers.

### Marker development

According to our previous work (Zhang et al. 2013), the target region of Ph-3 on the Heinz1706 reference genome (http://solgenomics.net) was selected to design PCR primers. Amplified PCR products from the parental lines were sequenced and analysed for polymorphisms in order to produce cleaved amplified polymorphic sequences (CAPS) or insert/deletion (InDel) markers.

### Construction and screening of BAC library

The bacterial artificial chromosome (BAC) library was generated using the Ph-3 donor species *S. pimpinellifolium* L3708 with restriction enzymes HindIII according to the previously described protocol (van der Voort et al. 1999). The BAC library was stored in 252 384-well microtiter plates, and all 384 clones in one plate were mixed to form a BAC pool. The BAC pool DNA was isolated by alkaline lysis method and screened with two markers TG591S and r2M1S that are closely linked to Ph-3. Afterwards, the single colony from the 384-well plates corresponding to the positive pool was identified using the same markers. DNA from the single positive colony was isolated and then tested with additional markers covering the Ph-3 region (Table 1).

### DNA sequencing and analysis

Sequence of the selected BAC clone harbouring the Ph-3 region was obtained by constructing a library of subclones (1–3 kb). Both ends of the subclones were sequenced using the ABI 3730xl platform and then assembled (BGI, Beijing, China). Putative genes in the BAC sequence were predicted with the online program FGENESH (http://linux1.softberry.com/) and protein functions were predicted with the InterProScan program (http://ebi.ac.uk/Tools/InterProScan/). Results were compared with

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### Table 1 Markers used for recombinant screening

| Marker names | Forward primer (5′–3′) | Reverse primer (5′–3′) | Type of marker |
|-------------|------------------------|------------------------|----------------|
| G2-4        | ATGCCACGACCATAAATC     | GACTGGGCTAATCAGCAAG    | CAPS with DraI |
| R1-3U       | AAAAGTTATCAGAGGGAATGA  | ATTGCAAGATCCTTGCACT    | Co-dominant SCAR |
| R2-3U       | TAGTGAACCGCTGATAAC     | CAATCTGGTGTGGAGAC      | dominant        |
| R2M1S       | GGAAATCTCCCGCTTCTATTT  | CGGTTGCAAACCTTAGACTCA  | Co-dominant SCAR |
| TG591S      | GCGAGACATAGCAAATC      | AACTGGGACGTTGTTGGA     | SNP             |
| M67-3       | TGCGAAATCTCTTGTTGTAAT  | CTACTGTTGGACTTGAGG     | CAPS with Sapl  |
| G7-5        | TGCCCTCTGAAGAGTGGT     | AAACGTTCGAGGGTTAT      | SNP             |
| G8-1        | CGCGGTTCGTGCGGGATTT    | AGCGTGTGGTGAGGTATT     | SNP             |
| M8-2        | AGGTGTCTCTATTCCATCA    | AATAGGGACCAATAGGAGG    | InDel           |

(Co-)dominant indicates that it is a (Co-)dominant marker. InDel indicates that this marker is derived from a short Insert/Deletion variation. CAPS cleaved amplified polymorphic sequence, SCAR sequence-characterized amplified region, SNP single-nucleotide polymorphism.
the Heinz1706 annotations derived from the International Tomato Annotation Group (ITAG2.3 version). ClustalW2 was used to align multiple sequences with default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Transformation of the Ph-3 gene into the susceptible S. lycopersicum cv. Moneymaker

A 8-kb fragment carrying the Ph-3 promoter, open reading frame (ORF) and terminator was amplified from the BAC plasmid B25E21 by PCR using the Phusion high-fidelity DNA polymerase (Thermo Fisher, Waltham, MA, USA) with primers Ph3EF3 (5′-taacctgcaggTTCAACACCATCTCTCATAGGGCG-3′) and Ph3ER3 (5′-atggegege-ctGGGGGCCTAGAAAAAGGTTG-3′). Two enzyme sites SfiI and AscI were added to the 5′ ends of forward and reverse primers, respectively. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced for confirmation. The resulting plasmid was digested with SfiI and AscI. The fragment containing the Ph-3 gene was then ligated into the binary vector pBINPLUS having a modified multiple cloning site. The positive gene was then ligated into the susceptible S. lycopersicum cv. Moneymaker was carried out as described by Huibers et al. (2013). Twenty-four regenerants that were capable of growing on kanamycin medium were transferred to the greenhouse. All kanamycin resistant regenerants were screened with the primer pair M67-3F (5′-TGCGAATCTTGTGGTAT-3′) and pBP-R2 (5′-AGGGAGAAAAGGTTG-3′), located in the 5′ ends of forward and reverse primers, respectively. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced for confirmation. The resulting plasmid was digested with SfiI and AscI. The fragment containing the Ph-3 gene was then ligated into the binary vector pBINPLUS having a modified multiple cloning site. The positive plasmid, named Ph13-2, was introduced into Agrobacterium tumefaciens strain AGL1 by electroporation.

Transformation of S. lycopersicum cv. Moneymaker was carried out as described by Huibers et al. (2013). Twenty-four regenerants that were capable of growing on kanamycin medium were transferred to the greenhouse. All kanamycin resistant regenerants were screened with the primer pair M67-3F (5′-TGCGAATCTTGTGGTAT-3′), located in the Ph-3 fragment) and pBP-R2 (5′-AGGGAGAAAAGGTTG-3′), located in the vector but within the T-DNA region).

Disease assay

Both whole-plant assay (WPA) and detached-leaf assay (DLA) were used for disease tests with P. infestans. The progenies of two recombinants (1-356 and 8-25) were tested by WPA as described by Zhang et al. (2013). The recombinants and Ph-3 transgenic plants were tested for P. infestans resistance through DLA as described by Vleeshouwers (1999). Three leaves of each plant were used and inoculated with P. infestans isolate T1,2,4 (Zhang et al. 2013). Two independent disease tests were performed for DLA.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using RNeasy plant mini kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). cDNA was diluted tenfold and used for real-time PCR (RT-PCR) with the Bio-Rad CFX96™ thermal cycler according to the protocol provided by the manufacturer. To detect the expression of R gene analogues (RGAs) in CLN2037B, samples were taken from three plants, and for each plant three leaves were pooled for RT-PCR. The primers R1eF1 (5′-GAAAAGGGATGCAGAACACCA-3′) and R1eR1 (5′-CGACAAACTTTGGTCGCGAAG-3′) located in ORF2, which produced a 181-bp fragment, were used to test the expression of ORF2. The primers used to check the expression of ORF3 were R2eF1 (5′-TTCTCTCTACTGACGTGCA-3′) and R2eR1 (5′-TCACACTTTTTGCCTTGG-3′), which produced a 164-bp fragment. For analysis of the Ph-3 expression level in the primary transgenic plants, the primers R2eF1 and R2eR1 were used. The tomato elongation factor 1α (EF1α) gene (Gene ID: 544055) was used as the internal reference in all analyses which was amplified with forward (5′-ATTGGAAACGGATATGCTCCA-3′) and reverse primers (5′-TTCCCTACCTGAAGCCCTGTA-3′). Gene expression level was calculated on the basis of the 2−ΔΔCt method (Livak and Schmittgen 2001).

Results

Fine mapping of the Ph-3 gene

Previously, Ph-3 was mapped into a 74-kb interval on the long arm of chromosome 9 (Zhang et al. 2013). In this region, eight genes were identified in the Heinz1706 reference genome (The Tomato Genome Consortium 2012). To further narrow down the Ph-3 interval, approximately 1,900 plants from two F2 populations (CLN2037B × 02393 and CLN2037E × 02393) and eight F2 populations (derived from CLN2037B × LA4084) were screened with two markers G2-4 and M8-2 flanking the Ph-3 gene (Table 1). Seven recombinants were identified and genotyped with additional markers located in between G2-4 and M8-2. Three leaves of each recombinant were inoculated with P. infestans isolate T1,2,4 through DLA. In two independent experiments, five recombinants (1-104, 4-35, 1-356, 7-111, 4-54) containing the S. pimpinellifolium L3708 introgression between markers G2-4 and M67-3 were resistant, while two recombinants (8-25 and 2-125) lacking this introgression were susceptible (Table 2). This result indicated that Ph-3 was located in between markers G2-4 and M67-3, a region of 41 kb in the Heinz1706 genome. The progenies of two important recombinants (1-356 and 8-25) were tested with P. infestans using the whole-plant assay. In the progeny of 1-356, two out of the 10 tested plants were susceptible, suggesting that the Ph-3 gene was located in the heterozygous region, upstream of the marker M67-3. All 27 progeny plants from 8–25 were

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susceptible confirming that the introgression between markers G2-4 and R2M1S did not carry the late blight resistance. Therefore, the \textit{Ph}-3 gene was delimited to a 26 kb region between markers \textit{r2M1S} and \textit{M67-3} based on the Heinz1706 reference genome.

**BAC library screening**

In the previous study, we have demonstrated that the \textit{Ph}-3 gene belongs to a CC-NBS-LRR gene family at the end of the long arm of chromosome 9. In the Heinz1706 genome, there are four members of this gene family present in the 74-kb interval where \textit{Ph}-3 is located (Zhang et al. 2013). We thus tried to amplify the full length of the homologous alleles in \textit{S. pimpinellifolium} l3708 (the donor of the \textit{Ph}-3 gene). Unfortunately, primers designed according to the Heinz1706 reference genome failed to amplify the full length of candidate homologs from the \textit{Ph}-3-carrying tomato lines. In order to obtain the sequence covering the \textit{Ph}-3 locus, the \textit{Ph}-3 donor \textit{S. pimpinellifolium} L3708 was used to construct a BAC library. The library consisted of 96,768 clones with an average insert size of 100 kb based on pulsed-field gel analysis of randomly selected clones. The library was thus estimated to represent approximately tenfold coverage of the L3708 genome.

Two PCR markers R2M1S and TG591S (Fig. 1), which were closely linked to \textit{Ph}-3, were used to screen the BAC library. A positive BAC pool B25 was identified from which a positive clone B25E21 was picked up. Subsequently, the full length of B25E21 was sequenced. The whole BAC sequence of B25E21 carries an insert of 73,671 bp from \textit{S. pimpinellifolium} l3708, corresponding to an interval of 101,456 bp in the Heinz1706 reference genome starting from Sl2.40ch09:66725592 and ending at Sl2.40ch09:66827013. The sequence alignment showed that the first 29 kb and the last 37 kb of BAC clone B25e21 were collinear with the reference sequence except two short deletions in the first 10 kb (Fig. 2). The major difference was the high variable region in the middle, starting at about 66,762 kb–66,795 bp based on the Heinz1706 genome.

**Candidate of \textit{Ph}-3**

In the previous study, we have demonstrated that \textit{Ph}-3 is an RGA of an NBS-type family (Zhang et al. 2013). In the Heinz1706 genome, the \textit{Ph}-3 interval carries four RGA
members (SlRGA1-SlRGA4). However, in S. pimpinellifolium L3708 genome, it contains only two RGAs (OrF2 and OrF3) (Fig. 3), which show different nucleotide identity to the four RGAs in the Heinz1706 genome (Fig. 3; Table S1). In the tomato line CLN2037B carrying the Ph-3 gene, the expression of OrF3 but not OrF2 was detected (Fig. S1). Since Ph-3 was mapped to a 26 kb interval between markers R2M1S and M67-3 (Table 2), a region harbouring only OrF3, the ORF2 was thus excluded to be the Ph-3 candidate.

The key recombinant 8-25 which resulted from recombination events in the RGAs region was analysed. This susceptible recombinant is heterozygous at the R2M1S locus while it is homozygous for the LA4084 allele at the TG591S locus. Both R2M1S and TG591S are located within ORF3. The progeny plants of the recombinant 8-25, which was homozygous for the CLN2037B allele at R2M1S locus and homozygous for the LA4084 allele at TG591S locus were selected and used to amplify the DNA fragment with the R2M1S forward primer and the TG591S reverse primer. Subsequently, this sequence was aligned with the alleles from S. pimpinellifolium L3708 and the susceptible parent LA4084. In this way, the crossing-over event of 8-25 was pinpointed between two SNPs (Fig. 4) which are 465 bp apart. Since all progeny plants of 8-25 were susceptible, it is very likely that the recombination event in 8-25 led to a non-functional chimeric OrF3, suggesting that ORF3 was the most likely candidate of Ph-3.

Complementation analysis

To analyse the function of the Ph-3 candidate gene, a fragment encompassing 3,565 bp upstream and 1,866 bp

Fig. 2 Comparison of the Ph-3-containing BAC sequence with the Heinz1706 reference sequence. The X-axis shows the sequence of BAC B25E21, and the Y-axis shows the corresponding Heinz1706 reference sequence. The sequences were analysed using dotter (v6.0.1) with a window size 10. Arrow points to the large picture of the variable region carrying RGAs

Fig. 3 Schematic of the microsynteny between the R gene clusters at the Ph-3 locus in S. pimpinellifolium L3708 and S. lycopersicum Heinz1706. The green arrows at the top show the R gene homologs in the L3708 BAC sequence, and the yellow arrows at the bottom indicate RGAs at the corresponding locus of Heinz1706. The transcriptional orientations are indicated by the direction of arrows. The orange, purple and blue lines linking the L3708 and Heinz1706 sequences indicate an identity above 95, 90–95, and 85–90 %, respectively.
downstream of ORF3 was amplified from BAC clone B25E21 and cloned into the binary vector pBINPLUS. The resulting plasmid was used for Agrobacterium-mediated transformation of the susceptible tomato cv. Moneymaker. In total, 14 independent transformants containing the Ph-3 gene were obtained and tested for resistance to P. infestans isolate T1,2,4. Among them, nine transgenic plants were resistant to P. infestans, while the remaining five plants were susceptible (Fig. 5; Fig. S2). Compared with Cln2037B, all resistant transgenic plants except CZ-T04 showed comparable or higher expression levels of the Ph-3 gene (Fig. S2). Therefore, the ORF3 under the control of its native promoter and terminator was sufficient to provide resistance to P. infestans in the susceptible Moneymaker plants, showing that ORF3 is the Ph-3 gene.

Structure of Ph-3

The Ph-3 gene (GenBank accession number: KJ563933) consists of one exon of 2,556 nucleotides, encoding a predicted polypeptide of 851 amino acids (Fig. 6). The deduced Ph-3 protein belongs to the CC-NBS-LRR class of plant R proteins. A predicted coiled-coil (CC) structure is located in the n-terminus between amino acids 63 and 84. Therefore, the entire n-terminus, from amino acid 1 till 150, is referred to as the CC domain. The nBS domain resides between residues 151 and 449, where the conserved nB-ArC motifs are present (van der Biezen and Jones 1998; Meyers et al. 2003). It is remarkable that the HD (H means histidine and D means aspartic acid) domain is located within the predicted lrr region, like the proteins encoded by R9a and Tm-2 (Jo 2013; lanfermeijer et al. 2003). The C terminal sequence only loosely fits the consensus for intracellular leucine-rich repeats (lrr), lxxlxxlxlxxC/nxx (where l represents leu, Ile, Val or Phe, n stands for Asp, Thr, Ser or Cys, and x is any amino acid) (van Ooijen et al. 2007). However, the consensus sequence for the β-sheet core (xxlxlxx) could be distinguished and totally 16 irregular lrr were found.

Among the cloned potato Rpi genes, Ph-3 shares high identity ranging 74.7–78.7 % to three chromosome-9-derived potato Rpi genes, Rpi-vnt1.1 from S. venturii, Rpi-mcq1 from S. mochiquense and R9a from S. demissum (Foster et al. 2009; Pel et al. 2009; Jones et al. 2009; Jo 2013) (Table S2). As shown by other studies (e.g. Jupe et al. 2013; Parniske et al. 1997), the lowest identity among
these Rpi proteins was found in the LRR domain. Taking Ph-3 and Rpi-vnt1.1 as an example, the identity in LRR domain is 63.3% while the CC and NBS domains show an identity of 90.7 and 91.4%, respectively. In addition, Ph-3 also shares high amino acid identity to the tomato mosaic virus resistance gene Tm-22 from S. peruvianum, which is located near the centromere of chromosome 9 (Table S2) (Lanfermeijer et al. 2003). All chromosome-9-derived proteins (Ph-3, Rpi-vnt1.1, Rpi-vnt1.2, Rpi-vnt1.3, Rpi-mcq1, R9a and Tm-22) belong to one clade (Fig. 7), which is distinct from other identified potato Rpi proteins.

**Discussion**

In a previous study, we have demonstrated that Ph-3 belongs to the NBS-LRR R gene cluster on chromosome 9 (Zhang et al. 2013). Unfortunately, the full length of candidate R gene homologs could not be amplified from the Heinz1706 reference genome. The failure in such a homology-based cloning was likely due to SNPs present in sequence of S. pimpinellifolium compared to the Heinz1706 reference genome (Fig. 2). We have therefore taken a map-based cloning approach for cloning of the Ph-3 gene, which is the first cloned tomato late blight R gene. Like the most cloned R genes, the Ph-3 gene also belongs to the NBS-LRR complex. Compared with the tomato Heinz1706 genome sequence, there is a deletion in...
the Ph-3 region of S. pinnipellifolium L3708 where RGAs are clustered. In Heinz1706, there are four RGAs while in L3708 there are only two RGAs. One of the two RGAs, ORF3, is confirmed to be the Ph-3 gene.

A hotspot carrying Rpi genes on chromosome 9 of Solanum species

It is well known that the NBS-LRR class of R genes is often clustered in the genome as a result of tandem and segmental duplications (Hubert et al. 2001; Leister 2004; McDowell and Simon 2006). Occasionally, all R genes in one cluster are functional, as is the case for R3 locus for late blight resistance in potato (Huang et al. 2005; Li et al. 2011b). In this study, however, only one RGA in the Ph-3 cluster contributes to the resistance for late blight. The Ph-3 gene is located at the end of the long arm of chromosome 9, a region carrying many Rpi genes in Solanaceae. In Solanum species, Rpi genes including Rpi-vnt1.1, Rpi-mcq1, R8, R9a, Rpi-edn2 and Rpi-dlc1, are located in this region (Pel et al. 2009; Smilde et al. 2005; Jo et al. 2011; Jo 2013; Verzaux 2010; Golas et al. 2010). Due to high variability of R gene clusters across species and lack of flanking sequences of these Rpi genes, it is hard to determine if all or any of these genes are orthologs of Ph-3. Nevertheless, Ph-3 exhibits highest identity to Rpi-vnt1.1, Rpi-mcq1 and R9a. These Rpi proteins are quite conserved in the CC and NBS domain (Jupe et al. 2013), while there is a high degree of amino acid variability in predicted solvent exposed residues of the LRR parallel β-sheet structure, a determinant of recognition specificity (Parmiske et al. 1997).

The changes of generating a gain of function allele by random mutation alone are extremely low (Parmiske and Jones 1999). Evolution of R genes is driven by gene duplication and unequal crossing-over followed by diversifying selection (Michelmore and Meyers 1998; Hulbert et al. 2001). For example, the presence of tandemly duplicated homologous sequences at the Cf-4/Cf-9 locus promote chromosome mispairing followed by unequal crossing-over or gene conversion events (Thomas et al. 1997). In the Ph-3 cluster, there are two and four RGAs in S. pinnipellifolium L3708 and S. lycopersicum Heinz1706, respectively. All RGAs in these two genomes share high identity with each other ranging from 87.8 % (SIRGA1 and SIRGA3) to 97.5 % (SIRGA3 and SIRGA4) (Table S1), which possibly promotes the unequal homologous recombination. Furthermore, we indeed showed that the Ph-3 allele in the recombinant 8-25 resulted from an unequal crossover between the Ph-3 gene and the susceptible allele, which led to a chimeric and non-functional RGA. It suggests that chromosomal rearrangements within R gene clusters do occur, resulting in the formation of a novel allele.

A combined use of tomato and potato Rpi gene to achieve durable resistance

Both tomato and potato are hosts of P. infestans. The resources of late blight resistance in tomato germplasm are less abundant than in the potato. So far, all tomato Rpi genes, which are useful for resistance breeding, are identified in the wild species S. pinnipellifolium (Bonde and Murphy 1952; Gallegly and Marvel 1955; Peirce 1971; Moreau et al. 1998; Black et al. 1996a, b; Chunwongse et al. 2002; Foolad et al. 2006, 2008; Merk et al. 2012; Merk and Foolad 2012). Although Ph-3 is widely used in tomato breeding, the resistance of Ph-3 has been overcome. Chunwongse et al. (2002) reported that four isolates were virulent on the Ph-3 donor L3708. Therefore, it is necessary to investigate if other wild relatives of tomato can provide novel monogenic Rpi genes conferring race-nonspecific resistance.

Durable disease resistance is the ultimate goal of many breeding programmes. Durable resistance has no particular genetic basis. It is a consequence of both the nature of resistance in the plant and the evolutionary potential of the pathogen (Michelmore et al. 2013). Some monogenic R genes, such as Lr34 in wheat, mlo in barley and other species, have proved durable over many years of agricultural use (Krattinger et al. 2013; Jørgensen 1992; Bai et al. 2008). For late blight, however, single R genes were quickly overcome in the field. Stacking of two or multiple Rpi genes can confer resistance to a broad and complementary set of isolates (Zhu et al. 2012, 2013). For stacking strategy, the knowledge of interaction between P. infestans (effectors) and host (R genes) is essential, which helps to evaluate the durability of R genes (Velessouders et al. 2008). The Ph-3 gene has high identity with two potato Rpi genes (Rpi-vnt1.1 and R9a) of which corresponding effectors are known (Pel 2010; Jo 2013). Whether Ph-3 recognizes these effectors is still not clear.

An alternative approach to manage late blight in tomato is to introduce potato Rpi genes into tomato. It has been reported that the potato Rpi genes Rpi-blb1, Rpi-blb2, R1, R3a, Rpi-vnt1.1, and Rpi-mcq1 were functional in tomato (van der Vossen et al. 2003, 2005; Jia et al. 2009; Foster et al. 2009; Jones et al. 2009). The Rpi-blb1 or Rpi-blb2 transgenic tomato plants not only showed resistance to P. infestans isolates from potato, but also to the isolates from tomato (van der Vossen et al. 2003, 2005; Jia et al. 2009), which illustrates the potential effectiveness of the employment of potato Rpi genes in tomato. Furthermore, we observed that the tomato line CLN2037B containing Ph-3 were resistant to multiple potato isolates (data not shown), suggesting that Ph-3 could protect potato from late blight. However, Oyarzun et al. (1998) observed a greater specificity of isolates for their first host than for their alternative
host. Also Vega-Sánchez et al. (2000) found that tomato and potato were attacked by two separate, host-adapted populations of P. infestans. Therefore, it is still unclear that the resistance to potato isolates is mediated by the Ph-3 gene or other host or pathogen factors. In addition, the Ph-3 gene provides only partial resistance (Zhang et al. 2013). Although complete resistance was achieved when the Ph-3 gene was highly expressed under its native promoter, an absolute correlation between gene expression level and resistance was not found (Fig. S2). Compared to CLN2037B, the Ph-3 gene was significantly higher expressed in three transgenic plants (T10, T09, T15) which showed no symptoms on the inoculated leaves. However, not all transgenic plants having a similar level of expression as the one in CLN2037B showed resistance. One possible reason is the difference at insertion locations of the Ph-3 gene. Alternatively, the expression of Ph-3 might be influenced by developmental stages and environments because leaves used for inoculation and RNA extraction were different. Thus, the resistance level in these transgenic tomato lines needs to be confirmed by testing their progenies. Further, transformation of Ph-3 into susceptible potato cultivars and analysis of their resistance level and spectrum will verify the potential effectiveness of the employment of Ph-3 in tomato breeding programmes.

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Conflict of interest All benefits in any form from a commercial party related directly or indirectly to the subject of this manuscript or any of the authors have been acknowledged. The authors of this manuscript declare that they have no conflict of interest.

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