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Rui Jiang[1,2,5], Jingcai Li[1,2,9], Zhendong Tian[2,3,4,5], Juan Du[2,3,5], Miles Armstrong[6,8], Katie Baker[6,8], Joanne Tze-Yin Lim[6,8], Jack H. Vossen[10], Huan He[2,3,4,5], Leticia Portal[7], Jun Zhou[1,2,5], Merideth Bonierbale[7], Ingo Hein[6,8*], Hannele Lindqvist-Kreuze[7*], Conghua Xie[1,2,5*]

[1] Key Laboratory of Potato Biology and Biotechnology, Ministry of Agriculture, P. R. China, Wuhan 430070, China

[2] National Center for Vegetable Improvement (Central China), Wuhan 430070, China

[3] Key Laboratory of Horticultural Plant Biology (HZAU), Ministry of Education, Wuhan 430070, China

[4] Potato Engineering and Technology Research Center of Hubei Province, Wuhan 430070, China

[5] Huazhong Agricultural University, Wuhan, Hubei 430070, China

[6] Cell and Molecular Sciences, The James Hutton Institute, Dundee, Scotland, DD2 5DA, UK

[7] International Potato Center, Apartado 1558, Lima 12, Peru

[8] The University of Dundee, Division of Plant Sciences at the James Hutton Institute, Dundee, DD2 5DA, UK

[9] School of Life Sciences, Huanggang Normal College, Huanggang, Hubei, 438000, China

[10] Wageningen UR Plant Breeding, Wageningen University and Research, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

*Corresponding authors. Tel: +86 27 87280969; Fax: +86 27 87286939.

E-mail addresses:

jiangrui828@webmail.hzau.edu.cn (Rui Jiang), lijingcai@hgnu.edu.cn (Jingcai Li), Tianzhd@mail.hzau.edu.cn (Zhendong Tian), juandu@mail.hzau.edu.cn (Juan Du), miles.armstrong@hutton.ac.uk (Miles Armstrong), katie.baker@hutton.ac.uk (Katie Baker), Joanne.Lim@hutton.ac.uk (Joanne Tze-Yin Lim), Jack.Vossen@wur.nl (Jack H. Vossen), 284624399@qq.com (Huan He), l.portal@cgiar.org (Leticia Portal), 26510955@qq.com (Jun Zhou), M.BONIERBALE@CGIAR.ORG (Merideth

Bonierbale), Ingo.Hein@hutton.ac.uk (Ingo Hein), h.lindqvist-kreuze@cgiar.org (Hannele Lindqvist-Kreuze), xiech@mail.hzau.edu.cn (Conghua Xie).

Running title: *R8* confers potato durable late blight resistance

Highlight:

Using genetics, genomics and diagnostic RenSeq, we demonstrate that the major NB-LRR gene *R8* explains the field resistance against potato late blight in QTL *dPI09c*.

Abstract:

Following the often short-lived protection that major nucleotide binding, leucine-rich-repeat resistance genes offer against the potato pathogen *Phytophthora infestans*, field resistance was thought to provide a more durable alternative to prevent late blight disease. We previously identified the QTL *dPI09c* on potato chromosome 9 as a more durable field resistance source against late blight. Here, the resistance QTL was fine-mapped to a 186 kb region. The interval corresponds to a larger, 389 kb, genomic region in the potato reference genome of DM and from which functional NB-LRRs *R8*, *R9a Rpi-moc1* and *Rpi_vnt1* have arisen independently in wild species. dRenSeq analysis of parental clones alongside resistant and susceptible

bulks of the segregating population B3C1HP, showed full sequence representation of *R8*. This was independently validated using long-range PCR and screening of a bespoke BAC library. The latter enabled a comparative analysis of the sequence variation in this locus in diverse Solanaceae. We reveal for the first time that broad spectrum and durable field resistance against *P. infestans* is conferred by the NB-LRR gene, *R8*, which is thought to provide narrow spectrum race-specific resistance.

Keywords: dRenSeq, field resistance, late blight, map-based cloning, potato, *R* gene

Abbreviations:

dpi: days post inoculation

dRenSeq: diagnostic RenSeq (Resistance gene enrichment sequencing)

QDR: quantitative disease resistance

Introduction

Potato (*Solanum tuberosum* L.), is the third most important food crop in the world after rice and wheat in terms of human consumption. More than a billion people worldwide consume potatoes, and global crop production exceeded 382 million metric tons in

2014 (FAO, 2017). *Phytophthora infestans*, the causal agent of late blight disease, is the most devastating pathogen of potato, causing losses of approximately $6.7 billion annually (Haas *et al.*, 2009). Preventative application of chemicals is currently being used to control this disease. However, excessive use of fungicides poses detrimental risks to human health and to the environment. Moreover, isolates can become insensitive to some of the commonly used agents (Goodwin *et al.*, 1996). Thus, the characterization, cloning and introgression into cultivars of natural resistance provide an environmentally benign alternative to chemical crop protection agents.

The wild species *S. demissum* has been used as a donor for single race-specific resistance (*R*) genes, which mediate complete resistance to *P. infestans* isolates carrying cognate avirulence protein (Lenman *et al.*, 2016). Nonetheless, a dynamic, repeat-rich genome enables the pathogen to often evolve rapidly (Haas *et al.*, 2009; Raffaele *et al.*, 2010) and to overcome host resistances through the emergence of new pathogen races (Fry *et al.*, 1997). Pyramiding multiple major *R* genes is considered as one sustainable strategy to maintain the resistance for longer (Zhu *et al.*, 2012; Haesaert *et al.*, 2015), while deploying quantitative disease resistance (QDR) in breeding programs has also been adopted (St Clair, 2010). QDR has been described as horizontal, incomplete, field, durable and broad-spectrum resistance by different authors owing to their interests and assumptions (Solomon-Blackburn *et al.*,

2007; Poland *et al.*, 2009). However, phenotypically, all resemble the same phenotype: reduced disease symptoms, but not the absence of them. It is thought that these resistances could be more durable as the evolutionary pressure to adapt is significantly decreased for the pathogens. Therefore, QDR has been favored by potato breeders after initial simple stacks of *R* genes were overcome in the 1960s as shown for *R1*, *R2* and *R3a* in the cultivar Pentland Dell (Hein *et al.*, 2009).

Since the 1980s, the International Potato Center (CIP) has developed durable late blight resistant potato germplasms by excluding the known major *R* genes from selected resistant sources. The strategy was meant to eliminate the interference of *R* genes, because they could potentially mask the underlying quantitative resistance in the process of selection (Landeo, 1989). Three selection steps were carried out with complex *P. infestans* races that were predicted to overcome the major *R* genes as well as race 0, and the resulting population was known as B3 (Landeo, 1989; Landeo *et al.*, 1995). To date, the B3 population has undergone three cycles of recurrent selection to improve agronomic traits whilst maintaining broad-spectrum late blight resistance (M. Gastelo, CIP; personal communication), and a number of cultivars have been released by CIP national partners (Ndacyayisenga, 2011). To understand the genetic and molecular mechanism of the quantitative late blight resistance in the B3 population, a dihaploid population, B3C1HP, which originates from the resistant B3

advanced clone 393046.7, was used to construct a genetic map. Five independent field assessments were conducted at two locations in Peru and a major QTL, *dPI09c*, was detected on chromosome 9. The QTL *dPI09c* explained between 14.5–83.3% of the disease variance in different environments (Li *et al.*, 2012).

Map based cloning has been widely used for potato late blight resistance gene cloning, such as *R1* (Ballvora *et al.*, 2002), *R2* (Lokossou *et al.*, 2009) and *RB* (Song *et al.*, 2003). Recently, with the development of high-throughput sequencing platforms, the potato genome has been sequenced (Potato Genome Sequencing Consortium, 2011; Aversano *et al.*, 2016), which can greatly accelerate the identification of genes of interest. A total of 755 NB-LRR genes have been identified and used as probes to establish resistance gene enrichment sequencing (RenSeq) platform (Jupe *et al.*, 2012; Jupe *et al.*2013) to promote the identification of *R* genes through bulked-segregation analysis. Combined with single-molecule real-time (SMRT) sequencing (SMRT RenSeq) (Witek *et al.*, 2016), or chemical mutagenesis (MutRenSeq) (Steuernage *et al.*, 2016), RenSeq has been utilized in cloning *R* genes in different species. RenSeq has also been applied as diagnostic tool, dRenSeq, to identify known *R* genes or their homologues (Van Weymers *et al.*, 2016). In this study, we report the molecular characterization of the *dPI09c* locus through map-based cloning, dRenSeq, allele mining and comparative genomics.

Materials and methods

Plant materials

For the fine mapping of the resistance QTL, an extended progeny of the B3C1HP population was generated using the same progenitors 301071.3 as the resistant maternal parent and 703308 as the susceptible male plant, as described by Li *et al*. (2012). Over 4000 potato true seeds were germinated *in vitro*. The seeds were sterilized with 1.5% (v/v) sodium hypochlorite solution for 15 min and rinsed three times with sterile distilled water. Seed germination was induced by incubating sterile seeds with 1000 ppm gibberellic acid for 18 hours in the dark followed by culturing of seeds in plastic tubes (Falcon 15 mL conical) containing 5 mL of MS medium (Murashige & Skoog, 1962). The plants were multiplied in tissue culture using standard growth media for potato. Three-week-old *in vitro* plantlets were transplanted in pots containing promix.

Nicotiana benthamiana was grown in the greenhouse under a 16h/8 h light and dark cycle at 24°C. Leaves from 5-week-old plants were used for experiments.

DNA extraction and recombinants screening

The genomic DNA of all the 4000 progenies was extracted from three-week-old plantlets with a fast and simple method described by Hosaka (2004). For the screening of recombinants, four markers flanking the QTL *dPI09c* region Rpi-svnt1_367, DMC42152bf, DMC42144af and At3g24160f2 were used (Li *et al.*, 2012) (Supplementary Table S1). The PCR and polyacrylamide gel running procedures were as described by Li *et al.* (2012).

Late blight resistance evaluation

Whole plant inoculations of the 106 recombinants was conducted in a greenhouse by spraying *P. infestans* sporangia onto all recombinant plants and using Amarilis and Cruza-148 as resistant controls as well as Desiree, Yungay and Tomasa as susceptible controls. There were six plants of each genotype and three plants of each control distributed following a randomized complete block design. Plants were 45 days old since emergence and *P. infestans* isolate PSR24 (Race 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 tested on Black differential set) was used for the infections. This particular isolate had been collected from susceptible plants of the $B3C1HP_{100}$ progenies in the field and purified, at a concentration of 750 sporangia ml^{-1}. The inoculation was done with a hand-held sprayer until run-off. After the inoculation, plastic tents were constructed to cover the plants and maintain almost 100% humidity. Humidity was

maintained high in the green house by automatic sprinkler system that switched on every 15 minutes. The temperature ranged between 17-24°C. The disease level was evaluated by estimating the percentage area of infection in each plant seven days after inoculation. For the analysis, the average infection percentage of each genotype was calculated and the progenies were divided in two groups, resistant and susceptible. As the resistance phenotype was quantitative, all progenies that scored a disease level of at least 30% or higher, were considered susceptible, and the genotypes with disease level less than this were considered as resistant.

Field assessment of the 106 recombinants was carried out in Oxapampa (12°34'05" South 75°24'23" West), a highland jungle agroecological zone in the Peruvian Andes with high endemic late blight pressure, where was considered as a "hot spot" for diversity of the *Phytophthora* (Gomez-Alpizar *et al*., 2007) with multi-races (Perez *et al*., 2001; Kaila, 2015). This is a recognized late blight resistance evaluation site of the International Potato Center's breeding program. During the assessment, the temperature varied from 8.8 to 30.4°C, with a mean of 18.4°C and the relative humidity varied from 38.4 to 100%, with a mean of 86.4%. The field trial was performed using the Alpha-Lattice design with 3 replications and each replication consisted of 10 plants per individual. Local variety Tomasa was used as susceptible control and 393046.7 (the original tetraploid resistance donor and parent of the

population) as a resistant control. Another susceptible variety Yungay was planted around the field to serve as an inoculum source. After the plants started to emerge the field was protected from late blight infection with weekly sprays of fungicide until all plants had fully emerged. After this the endemic infection was allowed to proceed, and the disease levels were evaluated weekly until the susceptible control (Tomasa) was 100% infected. The last disease evaluation (7th evaluation) was done at Dec. 8, 2014. The percentage of leaf area affected was used to calculate the area under the disease progress curve (AUDPC) (Jeger and Viljanen-Rollinson, 2001) for each genotype by using the midpoint rule method (Campbell and Madden, 1990).

For sequential agroinfiltration and detached leaf late blight assays in *N. benthamiana*, the third to fifth fully expanded leaves (counted from the uppermost leaf) of 5-week-old *N. benthamiana* plants were used for agroinfiltration. Two days post *Agrobacterium tumefaciens* infiltration (dpi), plants were infected with 10 µl sporangia of *P. infestans* isolate 88069 adjusted to a concentration of 1.5×10^5 sporangia ml^{-1}. The droplets of sporangia suspension were inoculated onto the abaxial side of detached *N. benthamiana* leaf within the agroinfiltration site. Disease symptoms were monitored for up to 12 dpi under natural and UV light. Three replicates were conducted with at least 12 leaves in each replicate.

Marker development

New PCR markers were developed according to all gene sequences of the Potato Genome Sequencing Consortium (http://solanaceae.plantbiology.msu.edu/) pseudomolecule v4.03 from marker At3g24160f2 (Chr09: 58728502-58728908) to the distal end of chromosome 9. Genomic DNA samples from both parents were used as templates to amplify the polymorphism determined by PCR product length with newly developed markers of *dPI09c*. DNA of 15 resistant (AUDPC 0) and 15 susceptible (the largest 15 AUDPCs) progenies (Supplementary Table S2), which performed no recombination in *dPI09c* region were selected to form resistance and susceptible pools, and were used as template to confirm the polymorphism. The PCR products amplified by the three flanking markers (3233-1，8384-1 and 8586-1) and 4 linked markers (jr38，5455-1，jr69 and jr78-2) were sequenced and aligned to the reference genome to verify their positions and for successive bacterial artificial chromosome (BAC) screening. An overview of the newly designed primers is listed in Supplementary Table S3. Polyacrylamide (19:1) gel electrophoresis was used to separate PCR products, followed by silver staining (Li *et al.*, 2012).

BAC library construction, screening and sequencing

The BAC library (BACGENE, Wuhan, China) was constructed with the DNA of 304413.40, one resistant clone of $B3C1HP_{100}$. The BAC library clones were individually picked and stored in 228 384-well microtiter plates, 384 clones in each plate were mixed to generate plate super pools. Three flanking markers 3233-1，8384-1，8586-1 and 4 linked markers jr38，5455-1，jr69，jr78-2 were used to screen for positive super pools. Afterwards, clones in each row and column within a single plate were mixed to form row and column sub-pools. The same markers were used to locate the positive clones.

Plasmids of the positive BAC clones (clone 119 and 122) were isolated with the QIAGEN Plasmid Midi Kit (Qiagen, Hamburg, Germany), and subsequently sequenced using PacBio RS II sequencing and assembled into one contig (Personalbio, Shanghai, China).

dRenSeq analysis

Genomic DNA of resistant parent 301071.3, susceptible parent 703308, resistant bulk, and susceptible bulk consisting of 27 resistant or susceptible progenies, respectively, were enriched using NB-LRR baits (Jupe *et al.*, 2013). Enrichment was

followed by paired-end Illumina MiSeq sequencing and diagnostic RenSeq (dRenSeq) analysis as described previously (Van Weymers *et al.*, 2016). For characterizing the late blight resistance via dRenSeq, two genes recently identified at the end of chromosome 9, *R8* (KU530153) (Vossen *et al.*, 2016) and *R9a* (Jo et al., 2015; https://www.google.com/patents/US20140041072), were added to the reference library used by Van Weymers *et al.* (2016).

Allele mining

To confirm the presence of the complete target gene in the B3C1HP population, resistant female plant 301071.3 and two resistant progenies of $B3C1HP_{100}$, 304413.40 and 304413.74, were subjected to PCR using *R8*-specific primers (R8-UTR_F and R8-UTR_R) (Supplementary Table S1) followed by cloning and sequencing. Ma*R8* (the potato late blight differential of the Mastenbroek differential set Ma*R1*–Ma*R11*; Kim *et al.*, 2012; Vossen *et al.*, 2016), from which *R8* was cloned, was used as positive control, while susceptible male plant 703308 and two susceptible progenies of B3C1HP, 304413.19 and 304413.89, were used as negative controls. Long-range PCR was conducted with Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing, China) to generate blunt-end PCR products. Adenine was added to both 3' ends, using Taq polymerase 2µl, PCR product 36µl,

10*buffer 5µl, 2mM dATP 5ul, incubating at 72°C for 40 min. Afterwards, PCR products were purified and cloned into pGEM®-T vector (Promega), and transformed to ElectroMAX™ DH10B competent cells (Invitrogen). At least eight positive clones of each genotype were sequenced and aligned with *R8* using the ClustalX 1.81 (Thompson *et al.*, 1997) and Genedoc (Nicholas *et al.*, 1997) analysis.

Vector construction and agroinfiltration

Avr8 (Jo, 2013) minus signal peptide was amplified from *P. infestans* isolate 88069 with attB sites to generate the entry clone, and recombined with pB7WGF2 for N-terminal EGFP fusion using gateway technology. Resistance genes *R8* and *R8-like* were amplified with primer pB7-R8 (Supplementary Table S1) and ligated into empty vector pB7WGF2 which had been digested with restriction enzyme B*sp*1407I. Recombinants were transformed into *A. tumefaciens* strain GV3101 competent cells. *R3a* and *Avr3a* (Armstrong *et al.*, 2005) plasmids harboring the late blight resistance gene *R3a* and its cognate avirulence gene *Avr3a*, respectively, were also transformed into GV3101 as a positive control for co-infiltration tests that elicit a strong Hypersensitive Response (HR), while empty vector pB7WGF2 was used as negative control (Armstrong *et al.,* 2005).

GV3101 strains with target constructs were grown in liquid YEB medium at 28°C overnight, bacterial cells were collected and re-suspended in modified MMA buffer (10mM MES, 10mM $MgCl_2$, and 200mM acetosyringone). Agrobacteria stains containing the constructs of interest were mixed and adjusted to a final OD_{600} of 0.6 and 0.3 for *R* gene and *Avr* gene, respectively. The mixed agrobacteria suspension was incubated at room temperature for 2h in the dark before infiltration. After the incubation, a needleless syringe was used to infiltrate the agrobacteria suspension to a diffusion diameter of 1-1.5cm area through the abaxial leaf surface. HR responses were monitored five days after co-infiltration.

Comparative genome analysis

The BAC sequences of clones 122 and 119 span together the entire QTL *dPI09c* as defined by markers 3233-1/jr38 towards the centromeric part of LG 9 and 8384-1/8586-1 towards the distal end of the chromosome. Homologous representative sequences of the *dPI09c* interval from different species including potato DM1-3 516 R44 (Potato Genome Sequencing Consortium, 2011), 304413.40 (Resistant progeny used in this study), Ma*R8* (Vossen *et al.*, 2016), and tomato *Solanum lycopersicum* (Sato *et al.*, 2012) as well as *S. pennellii* (Bolger *et al.*, 2014) were selected for a comparative genomic study. Repeat stretches of ambiguous nucleotides (poly Ns) were removed resulting in sequences of 326,207 bp in length for

DM, 310,320 bp for *dPI09c*, 174,573 bp for *R8*, 301,334 bp for *S. lycopersicum* and 557,425 bp for *S. pennellii* (Supplementary Data S1).

Sequences were aligned using progressive Mauve algorithm (Darling *et al.*, 2004) in the program Geneious (version 10.2) using default condition (Automatically calculate seed weight; Compute Locally Collinear Blocks (LCBs); Automatically calculate the minimum LCB score; Full alignment using Gapped Aligner MUSCLE3.6).

Results

Fine mapping of the QTL *dPI09c*

Initial mapping carried out in the population $B3C1HP_{100}$ placed the QTL *dPI09c* within the proximity of the marker DMG400031529 (Li *et al.*, 2015), which resides near *R* gene clusters with homology to *Tm-2²* and *Sw-5* on potato linkage group 9 (Jo *et al.*, 2011; Jupe *et al.*, 2012; Li *et al.*, 2012; Jo *et al.*, 2015). To fine map the resistance, a larger population comprising 4000 additional clones ($B3C1HP_{4000}$) was assessed with initially identified markers Rpi-svnt1_367, DMC42152bf, DMC42144af and At3g24160f2 which resulted in 106 recombinants (Fig. 1, represented by Rec 1 and 2). Further resistance assessment of these recombinants with *P. infestans* isolate PSR24 in whole-plant greenhouse tests revealed an approximate 1:1 segregation

ratio of resistant recombinants which displayed less than 30% of leaf area infection with late blight and susceptible progenies with more than this. Field tests revealed a similar 1:1 segregation for resistance and susceptibility (Supplementary Table S2), which indicated a single dominant gene is concealed in this QTL. Additional markers (Supplementary Table S3) were developed towards the end of chromosome 9 based on the PGSC v4.03 pseudomolecule sequence (http://solanaceae.plantbiology.msu.edu/) at positions 58.72Mb to 61.40Mb. We identified one recombinant between markers STMput157a37146 and 3233-1 (Fig. 1, Rec 3), and two recombinants between markers 3233-1 and 8384-1 (Fig. 1, Rec 4 and 5). Furthermore, markers jr38, 5455-1, jr69 and jr78-2 were linked to the resistance. This ultimately narrowed the locus for resistance QTL *dPI09c* to a 389kb interval of the DM1-3 pseudomolecule sequence flanked by markers 3233-1 and 8384-1.

Functional *R8* was identified in *dPI09c* by dRenSeq

The fine mapping of the resistance in the $B3C1HP_{4000}$ population placed the QTL *dPI09c* in a genomic region that is known to contain a number of functional NB-LRRs such as *R8*, *R9a*, *Rpi_moc1* and *Rpi_vnt1* (Smilde *et al.*, 2005; Foster *et al.*, 2009; Pel *et al.*, 2009; Jo *et al.*, 2015; Vossen *et al.*, 2016). To ascertain if a known NB-LRR

gene could explain the resistance in the *dPI09c* QTL, we conducted a dRenSeq analysis (Van Weymers *et al.*, 2016). Genomic DNA of resistant parent 301071.3, susceptible parent 703308, bulks consisting of 27 resistant and 27 susceptible progenies of the B3C1HP$_{100}$ population were enriched using NB-LRR baits (Jupe *et al.*, 2013). Reads were mapped, using a high stringent 0.5% mismatch rate, against nine functional late blight NB-LRR genes including *Rpi-blb1*, *Rpi-blb2*, *R1*, *R2*, *R3a*, *R3b*, *R8*, *R9a*, and *Rpi-vnt1.1*. Under these conditions, the RenSeq reads only map to the reference set containing functional NB-LRRs if the reads have a maximum of one SNP in 200 bp of sequence compared to the reference. Mapping results demonstrated that the reads of the resistant parent and resistant bulk generated full coverage of *R8*, while only partial coverage was achieved using reads from the susceptible parent or the susceptible bulk (Fig. 2). The read depth is an important indication of the completeness of the coverage, as a partial coverage in susceptible parent and bulk indicates that some parts of the functional gene *R8* are conserved. These results strongly suggest that *R8* could be a main contributor towards the function of *dPI09c* for late blight resistance.

The presence of *R8* was confirmed by allele mining

To confirm the presence of a complete and intact *R8* gene in the $B3C1HP_{100}$ population, *R8*-specific primers (Supplementary Table S1) were utilized to amplify a 7kb fragment that encompasses functional *R8* (including coding and regulatory sequences). We included the susceptible male parent and two susceptible progenies (304413.19 and 304413.89) as negative controls, and Ma*R8* as a positive control. As expected, all susceptible plants did not yield the *R8*-specific amplicon (Supplementary Fig. S1). The PCR products of resistant female parent and two resistant progenies were cloned and sequenced. Our analysis confirmed that all clones contained the *R8* gene and no sequence variation was identified (data not shown).

R8 and functional *R8-like* resistance is found in diverse breeding material and wild species

To ascertain if *R8* is also present in additional late blight resistance resources, we PCR tested previously identified sources of late blight resistance that were obtained following late blight assays with highly aggressive *P. infestans* isolates UK3928A in the UK (Van Weymers *et al.*, 2016) and HB14-2 and HB16-2 collected in infected fields in the Hubei province of China. Thirty-two out of 242 tested genotypes showed resistance to the pathogens, with three susceptible cultivars (Yungay, E-Potato 3 and Huashu 1) being utilized as negative controls (Supplementary Table S4). The results

showed that twenty-one resistant plants putatively contained *R8* as they amplified the expected 7kb fragment. This included fifteen progenies descending from the B3 population, four cultivars (06HE13-1, 08HE171-1, 08HE171-6 and E-Potato 5), and two wild species (*S. phureja* accession IVP196-2 and *S. demissum* accession CT9-4). As expected, none of the susceptible plants yielded the *R8*-specific amplicon. PCR products of seven out of the fifteen progenies from the B3 population and all additional putative *R8*-containing genotypes were cloned and at least eight recombinant clones from each plant were sequenced. There was no sequence variation in *R8* alleles except for the wild species, *S. phureja* accession IVP196-2 and *S. demissum accession* CT9-4. In *S. phureja* IVP196-2, several insertions and deletions in the promoter were evident alongside many SNPs and a premature stop codon which results in a pseudogenised gene (Supplementary Fig. S2). *S. demissum* accession CT9-4 has a complete coding sequence like *R8* (Fig. 3A) except two non-synonymous SNPs at position 482 and 1051 in Solanaceace domain (SD) that changed the amino acids from Ile to Arg and from Phe to Leu, respectively (Fig. 3B) and was subsequently referred to as *R8-like*.

To investigate whether *R8*-like is a functional *R* gene, it was transiently co-expressed with the cognate avirulence gene of *P. infestans*, *Avr8*, in the model Solanaceae plant *Nicotiana benthamiana*, as shown by Vossen *et al.*, 2016. Co-infiltration-specific cell

death, which is indicative of a recognition response, was evident upon co-infiltration of resistance gene and avirulence gene like the positive control *R3a*/*Avr3a*, while the empty vector control pB7WGF2 showed no such phenotype. Importantly, this response between *R8-like* and *Avr8* was phenotypically not distinct from the cell death that was elicited after the co-infiltration of *R8* with *Avr8* five days post infiltration (Fig. 3C). Five-week-old *N. benthamiana* leaves transiently expressing *R8* and *R8-like*, were challenged with *P. infestans* isolate 88069. *R8-like* was demonstrated to be a functional *R* gene as it efficiently stopped colonization by *P. infestans* (Fig. 3D).

Genomic analysis of *dPI09c*

In order to investigate the genomic organization of the *dPI09c* locus, a resistant progeny of $B3C1HP_{100}$, 304413.40, was used to construct a BAC library. The library consisted of 87,552 recombinant clones with an average insert size of 110 kb, thus covering the genome 10 fold. The BAC library was screened with seven PCR markers of the 389kb region, and in total, seven positive BAC clones were identified (Fig. 1). The BAC clones 122 and 119 fully covered the *dPI09c* interval, and were subsequently sequenced. The assembled contig is 310.32 kb in length. Importantly, the contig contains the entire *dPI09c* interval flanked by markers 3233-1 and 8384-1

which is 186 kb in length and therefore shorter than the 389 kb region predicted in the potato reference genome.

Synteny analysis among homologous representative sequences of the *dPI09c* interval from potato DM (Potato Genome Sequencing Consortium, 2011), 304413.40 (Resistant progeny used in this study) and Ma*R8* (Vossen *et al.*, 2016), and tomato *S. lycopersicum* (Sato *et al.*, 2012) as well as *S. pennellii* (Bolger *et al.*, 2014), demonstrated rearrangements between all the haplotypes from different species in this interval, while a high homology between both Ma*R8* and 304413.40 was depicted (Fig. 4). Genomic sequence comparison between Ma*R8* and 304413.40 revealed that the same fragment has been introduced into different cultivars, suggesting that the resistance *dPI09c* is conferring might be derived from a common *S. demissum* source. Nevertheless, there is a slight difference between the two *R8* containing haplotypes at both BAC ends. The two analogues of *R8* in the front end of Ma*R8* BAC and 304413.40 BAC shared less than 40% sequence identity, and the similarity between the flanking sequence is very low as well (data not shown). This suggests either this is a breaking point of recombination in different haplotypes, or an incomplete sequencing of the haplotype. *R8* paralogue copy numbers varies in haplotypes from different species, with seventeen in DM, ten in 304413.30 and Ma*R8*, four in *S. lycopericum* and three in *S. pennellii*. However, *R8* sequence is somewhat

conserved comparing between the species. These results suggested that *R8* progenitors are most likely ancient genes that have undergone distinct evolution in different species.

Discussion

In a previous study, we have identified the late blight quantitative resistant QTL *dPI09c* (Li *et al.*, 2012) as a major QTL located at the end of the chromosome 9. Here, we have taken a map-based cloning approach, to narrow the genetically defined interval to a 389kb fragment with new markers 3233-1 and 8384-1 designed based on the reference genome of potato (Fig 1). In this region an array of late blight *R* genes including *Rpi-moc1* (Smilde *et al.*, 2005), *Rpi-vnt1* (Foster *et al.*, 2009), *R8* (Jo *et al.*, 2011), *R9a* (Jo *et al.*, 2015) and *Ph-3* (Zhang *et al.*, 2014) have been reported. Using diagnostic RenSeq method (dRenSeq), we identified the full coding sequence of *R8* in the resistant progenitor and the bulk consisting of resistant progenies. (Fig. 2). Allele mining confirmed that the resistance gene concealed in *dPI09c* is identical to *R8* of *S. demissum* (Vossen *et al.*, 2016). This was further confirmed by BAC library screening and sequencing, which also enabled a comparative genome study and indicated that this interval is conserved in other Solanaceae haplotypes.

This study provides clear evidence that a single major disease resistance gene can explain QTLs as has previously been speculated. Indeed, cultivars such as Sarpo Mira display field resistance-type responses to late blight and are now known to also contain *R8* but in combination with other major *R* genes (Rietman *et al.*, 2012 and Vossen *et al.*, 2016). B3C1HP population plants containing *R8* generally show expanding lesions in detached leaf assay (data not shown) and in B3 population the resistance in severe epidemics is consistent with what had been considered a quantitative type (Landeo *et al.*, 1999; Lindqvist-Kreuze *et al.*, 2014). CIP's B3 population incorporated a battery of resistant resources, including *S. demissum*-derived advanced sources of population A, native cultivars from *S. phureja*, *S. andigena* adapted to long days (Neotuberosum) and combined materials of *S. acaule*, *S. bulbocastanum* and *S. tuberosum* (ABPTs) through four-way hybrids (Landeo, 1989). An attempt was made to rule out major *R* genes in this population by selecting only progenies that were not fully resistant three decades ago (Landeo *et al.*, 1995). The quantitative resistance phenotype of B3C1HP is probably the reason why the responsive gene of *dPI09c*, *R8*, was retained in the B3 population after stringent screenings. Thus, this study significantly shows how difficult it can be to select progenies against the presence of major *R* genes. Whether minor contributors to *R8* reside in the genetically defined interval could be studied in the future as *R8*-based

resistance is highly dependent on the genetic background as described by Vossen *et al*. (2016) and in our detached leaf assay (data not shown). Hence, mining the *R8* gene stability and functionality regulator or other defense-related gene could also assist with the resistance breeding program in the future.

Some quantitative disease resistances (QDRs) have been identified to co-localize with major *R* gene locus. Three QTLs conferring resistance to powdery mildew *Oidium lycopersici* were found adjacent to qualitative loci, with *Ol-qtl1* on chromosome 6 in the same region as the *Ol-1*, *Ol-qtl2* and *Ol-qtl3* on chromosome 12 in the vicinity of the *Lv* locus conferring resistance to another powdery mildew species, *Leveillula Taurica* (Bai *et al*., 2003). It has been reported that potato late blight QDR on chromosome 5 co-localizes with *R1* (Collins *et al*., 1999; Beketova *et al*., 2006). However, it is likely that the quantitative resistance on chromosome 5 is not caused by QDR but by maturity type which is linked to *R1* (Collins *et al*., 1999; Beketova *et al*., 2006). In addition, the effects of "defeated" or "weak" *R* genes have been reported in many plants with quantitative disease resistance (Poland *et al*., 2009; Kou and Wang, 2010; St Clair, 2010; Roux *et al*., 2014; French *et al*., 2016). For example, the rice bacterial blight disease resistance gene *Xa4* has been regarded as a "defeated *R* gene" which confers resistance to multiple strains of *Xanthomonas oryzae pv. oryzae* (*Xoo*) (Li *et al*., 1999). In potato, QDR to late blight has been found durable in B3

population, Sarpo Mira, Stirling and other cultivars (Solomon-Blackburn *et al.*, 2007; Rietman *et al.*, 2012; Lindqvist-Kreuze *et al.*, 2014). In the present study, the cloning of *R8* provides strong evidence that quantitative resistance can be caused by NB-LRR gene, which is normally thought to be responsible for qualitative resistance. Besides, *S. demissum* differential Ma*R8*, Ma*R9* and Ma*R10* have also shown broad-spectrum and quantitative resistance in the field (Bradshaw *et al.*, 2006; Jo *et al.*, 2011; Xu *et al.*, 2013; Jo *et al.*, 2015), which suggests that when novel field resistance is identified, we must be careful with the assumption made about the molecular basis of such resistance and we cannot rule out major *R* genes as the main contributors. Helpfully, with the recently developed dRenSeq method, field resistance of potato materials can be quickly detected to check if known *R* genes existed, which is an efficient way to avoid time-consuming map-based cloning. Also, dRenSeq has a potential not only to investigate late blight resistance genes but also to an expansion of other significant traits, like, potato resistance to cyst nematodes, viruses and bacteria.

NB-LRR genes are well known to be clustered in plant genome by tandem and segmental duplications (Mcdowell and Simon, 2006). Functional homologs have been confirmed in late blight *R* gene clusters like *R2*, *R3a, R3b* and *Rpi-vnt1* locus (Huang *et al.*, 2005; Foster *et al.*, 2009; Lokossou *et al.*, 2009; Pel *et al.*, 2009; Li *et al.*, 2011;

Lenman *et al.*, 2016). In this study, we dissected that *R8* is located in such an *R* gene cluster. A functional *R8* homolog as defined by *R8-like* in *S. demissum* with two non-synonymous amino acid mutations (Fig. 3) was identified. These could result from random point mutation in different *S. demissum* accessions, or be a consequence of pathogen selection pressure in natural environments that might lead to novel effector recognition, which is not clear at present. Nonetheless, sequence alignment revealed that a similar fragment has been introduced to Ma*R8* and 304413.40 from *S. demissum* (Fig. 4), suggesting that scientists might exploit the same *S. demissum* resources (Black *et al.*, 1953; Malcolmson and Black, 1966) for resistance breeding against late blight. However, the BACs of 304413.40 for sequencing only contain the resistant haplotype derived from the resistant female parent 301071.3, little is known about the sequence of the susceptible one. There were no amplicons obtained when amplifying *R8* in susceptible plants. Therefore, difference between the resistant and susceptible haplotypes is speculated to be the sequence variations in the 5'UTR or the 3'UTR of *R8*, as the *R8*-specific primers were designed to amplify the full length of *R8* only. How *R8* might have evolved in the *dPI09c* locus between the two haplotypes remains to be established if the susceptible BAC clones could be identified and sequenced. Genomic comparison among Solanaceae species showed significant rearrangements in the *dPI09c* interval.

However, R8 shares 83.3% identity with its ortholog Sw-5b in tomato (Vossen *et al.*, 2016) and is relatively conserved compared to other analogues in Solanaceae. These findings suggest that *R8* analogues probably originated from the same ancestor and underwent distinct evolution in response to diverse challenges.

The *R8* gene was identified in some resistant potato cultivars (Table S4) indicating its feasibility in the resistance improvement to cope with unexpected environmental and pathogen changes. Single resistance gene with quantitative feature in resistance, has been observed to fight against a broad range of pathogens (Roux *et al.*, 2014), is considered as generalists. *Sw-5b*, the ortholog of *R8* in tomato (Brommonschenkel *et al.*, 2000; Spassova *et al.*, 2001), which has the Solanaceae domain (SD) (Mucyn *et al.*, 2006; Chen *et al.*, 2016), is a versatile *R* gene and confers broad resistance against tospoviruses, including *Tomato spotted wilt virus* (TSWV), *Groundnut ring spot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Boiteux *et al.*, 1993; Bendahmane *et al.*, 2002). Mi-1, also containing the SD domains which is thought to have dual regulatory roles of activating the Mi-1 resistance protein (Lukasik-Shreepaathy *et al.*, 2012), is responsible for the resistance against root-knot nematodes, whitefly and aphids (Rossi *et al.*, 1998; Vos, 1998; Nombela *et al.*, 2003). Whether *R8* can bring resistance to other pathogens like these SD encoding *R* genes,

and whether the mechanism underlying the quantitative resistance is due to the dynamic allele variation (Fig. 3) should be an intriguing area for further studies.

It has been argued that the *R* genes stacking is by far the best strategy to improve late blight quantitative resistance in the field (Stewart *et al.*, 2003; Zhu *et al.*, 2012). We know that some of the major *R* genes are more likely to be defeated as a result of strong selection pressure for the cognate fast evolving effectors, for instance, the evading form of Avr3a^{EM} (Armstrong *et al.*, 2005), the truncated Avr4 (van Poppel *et al.*, 2008) and the reduced expressed Avrvnt1 (Pel *et al.*, 2010). However, *R8*, *R9a*, *R10* and *Rpi-blb1* are still maintaining their quantitative resistance. *R* genes that confer quantitative disease resistance to late blight are more likely to be durable, which are presumed to have less selection pressures on pathogens and can be good candidates for *R* gene stacking. This novel way of stacking resistance genes could be a great achievement for breeding community to maintain the durability of potato resistance against late blight and increase the life expectancy of potato cultivars. Also, several of these genes stackings could be technically possible to induce high level resistance. Molecular markers are needed for every contributing QTL, including functional markers, like R/Avr gene responses, to help a rapid and precise selection of the resistance in breeding lines.

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Figure legends

Fig. 1 Fine mapping of *dPI09c* in population B3C1HP. Marker names and chromosomal positions are presented above and below the physical map, respectively. Marker names in red represent the markers developed in our previous study (Li *et al.*, 2012; Li *et al.*, 2015), markers in black are developed in this study. The transition of black and white rectangle indicates the cross-over happened in the progenies. R and S represent resistance and susceptible phenotype, respectively. Rec means recombination. The vertical dash line in blue indicates that the resistance of *dPI09c* has been narrowed to the interval between 3233-1 and 8384-1. Red boxes show the marker position. Chromosome walking identified seven BAC clones could cover the *dPI09c* interval. Two blue rectangles indicate BAC clones that can fully cover this interval.

Fig. 2 dRenSeq analysis on resistant and susceptible parent and bulks of population $B3C1HP_{100}$. Coverage of nine functional *R* genes and read depth converted to log10 scale are depicted in x-axis and y-axis. Light green and dark green curves represent resistant parent and bulk, red and scarlet curves represent susceptible ones, respectively. A high stringent mismatch rate (0.5%) is used for read mapping.

Fig. 3 The point mutations of *R8-like* in *S. demissum* does not change its function. (A) Schematic of R8 protein. SD, Solanaceace domain; CC, coiled-coiled domain;

NB-ARC, nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 domain; LRR, leucine rich repeat domain. (B) The alignment of *R8* and *R8-like* on the two point mutation fragments. The mutated bases are shaded in blue. (C) Transient overexpressing R8-like/Avr8 shows a similar level of HR as R8/Avr8. R3a/Avr3a was used as positive control, and empty vector (EV) pB7WGF2 negative control. The image was a representative of three biological repeats taken at 5 dpi under natural and UV light. (D) The leaf images under natural and UV light show the *P. infestans* colonization on *N. benthamiana* 12 dpi.

Fig. 4 Genomic comparison of *dPI09c* interval among potato DM1-3 516 R44 (Potato Genome Sequencing Consortium, 2011), 304413.40 (Resistant progeny used in this study) and Ma*R8* (Potato late blight differential of Mastenbroek differential set Ma*R1*–Ma*R11*; Vossen *et al.*, 2016), and tomato *Solanum lycopersicum* (Sato *et al.*, 2012) and *S. pennellii* (Bolger *et al.*, 2014). The big blocks in different color show the homology of the genome. The small green rectangles beneath the big blocks represent *R8*, blue ones are *R8* analogue, forward and reverse direction of the analogues are indicated by the upper rectangles and lower ones, respectively. Sequences were aligned using progressive Mauve algorithm (Darling *et al.*, 2004) in the program Geneious (version 10.2) with default settings.

Fig. 1

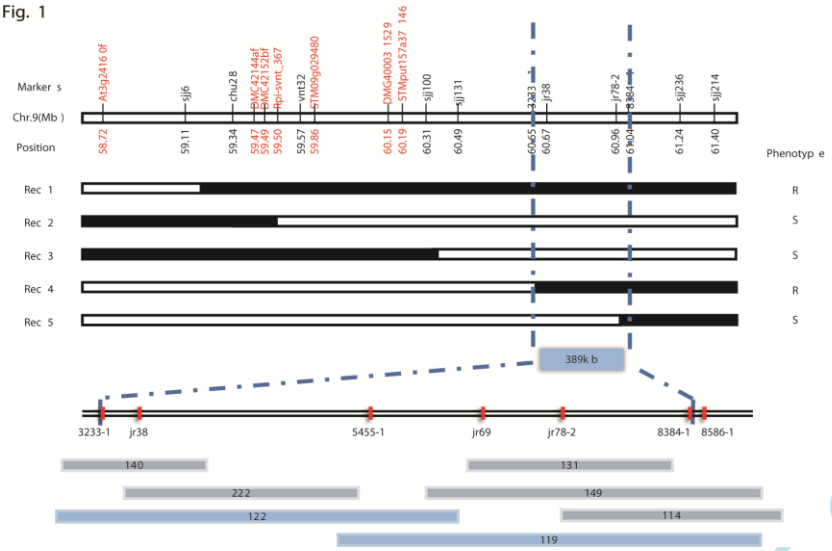

Fig. 2

Fig. 3

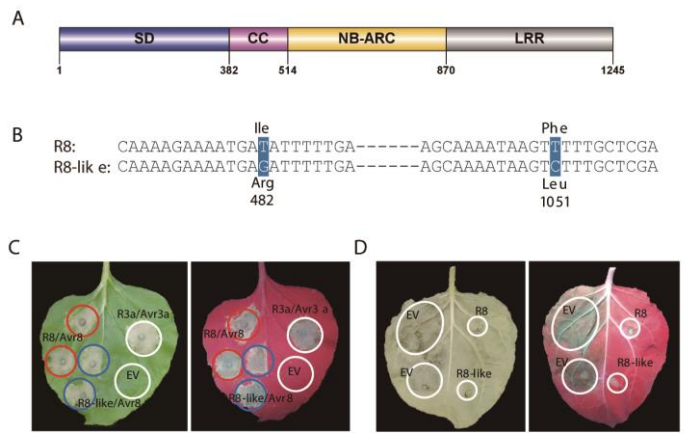

Fig. 4