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ORIGINAL PAPER

Comparative next-generation mapping of the *Phytophthora infestans* resistance gene *Rpi-dlc2* in a European accession of *Solanum dulcamara*

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Abstract *Phytophthora infestans*, the causal agent of late blight, remains the main threat to potato production worldwide. Screening of 19 accessions of *Solanum dulcamara* with *P. infestans* isolate Ipo82001 in detached leaf assays revealed strong resistance in an individual belonging to accession A54750069-1. This plant was crossed with a susceptible genotype, and an F_1 population consisting of 63 individuals was obtained. This population segregated for resistance in 1:1 ratio, both in detached leaf assays and in an open-field experiment. Presence of the formerly mapped *Rpi-dlc1* gene as the cause of the observed segregating resistance could be excluded. Subsequently, AFLP analyses using 128 primer combinations enabled identification of five markers linked to a novel resistance gene named *Rpi-dlc2*. AFLP markers did not show sequence similarity to the tomato and potato genomes, hampering comparative genetic positioning of the gene. For this reason we used next-generation mapping (NGM), an approach that exploits direct sequencing of DNA (in our case: cDNA) pools from bulked segregants to calculate the genetic distance between SNPs and the locus of interest. Plotting of these genetic distances on the tomato and potato genetic map and subsequent PCR-based marker analysis positioned the gene on chromosome 10, in a region overlapping with the *Rpi-ber/ber1* and *-ber2* loci from *S. berthaultii*. Pyramiding of *Rpi-dlc2* and *Rpi-dlc1* significantly increased resistance to *P. infestans*, compared with individuals containing only one of the genes, showing the usefulness of this strategy to enhance resistance against *Phytophthora*.

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Introduction

The oomycete *Phytophthora infestans* is the causal agent of late blight, the most important disease of cultivated potato (Fry 2007). Dynamic evolution of the pathogen is a major hurdle in introducing genetic resistance of practical value (Wastie 1991). However, the secondary gene pool of wild *Solanum* species offers a huge spectrum of resistance, and a number of valuable resistance genes have already been isolated from them (Jo et al. 2011). A plant known for a long time to be a host for *P. infestans* is *Solanum dulcamara* L., commonly named bittersweet or climbing nightshade (deBary 1876).

S. dulcamara is one of the ~1,500 species of the cosmopolitan genus *Solanum* L. (D'Arcy 1991; Weese and Bohs 2007). It belongs to the section *Dulcamara* (Moench) Dumort. of subgenus *Potatoe* that also includes the cultivated potato and its wild relatives (Child and Lester 2001). *S. dulcamara* is a diploid, perennial plant. It is one of few native Solanaceae present in Europe, although during the past few centuries it has been naturalized in many parts of the world. Bittersweet is often found in the vicinity of commercial potato fields where it has been hypothesized to serve as a reservoir for pathogens harmful for potato production, such as *Ralstonia solanacearum* (Smith) Smith, a causal agent of bacterial wilt (Olsson 1976; Elphinstone et al. 1996; Janse 1996).

Although *S. dulcamara* acts as a host for *P. infestans*, even favourable weather conditions for late blight only result in sporadic infections (Cooke et al. 2002; Flier et al. 2003a; Dandurand et al. 2006). Investigation of the nature of this possible resistance in the European population of *S. dulcamara* led to the identification and mapping on chromosome 9 of the *Rpi-dlc1* locus (Golas et al. 2010a). Still, despite the environmental impact of *S. dulcamara*, especially in North-western Europe, very little research was done on this plant species (Curtis et al. 2000). Indeed, the lack of genetic and molecular data hampered research, which relied mainly on information obtained from related species such as tomato and potato. Recently, progress in efficiency and a decrease of costs have allowed next-generation sequencing to be applied for linkage-disequilibrium mapping, an approach named next-generation mapping (NGM) (Lister et al. 2009; Austin et al. 2011). When working with non-model species, the combination of NGM with comparative sequence analyses of well-characterised related species can enable the identification of the gene linked to the trait of interest and its concurrent positioning on the physical and genetic maps. Moreover, as 'by product', this approach generates large amounts of sequence information that is valuable for performing research on species lacking molecular data.

In the present study we describe the identification and genetic mapping of a novel gene, named *Rpi-dlc2,* which confers resistance against *P. infestans* in *S. dulcamara*. Comparative genomics with data from potato and tomato allowed this locus to be assigned to *S. dulcamara* chromosome 10, in a region that is known to harbour the resistance genes *Rpi-ber* (Rauscher et al. 2006), *Rpi-ber1* and *Rpi-ber2* in *S. berthaultii* (Park et al. 2009). Resistance evaluation under field conditions of populations containing one or both of the two resistance genes, *Rpi-dlc2* and the previously mapped *Rpi-dlc1* gene, revealed additive effectiveness of the two genes.

Materials and methods

Plant material

Seeds of 15 *S. dulcamara* accessions were collected from the Radboud University Experimental Garden and Genebank (The Netherlands). In addition, the collection included four genotypes (each treated as an accession) obtained as cuttings from plants growing in natural settings at the Thames in Great Britain in the spring 2005. Individual number 2 from accession 944750001, which is susceptible to late blight, was used as control in resistance assays and as pollen donor (Table 1). Crosses were conducted under greenhouse conditions. Just before opening, flowers were emasculated and hand pollinated the next day. Six weeks after pollination, seeds were extracted from mature berries and stored in paper bags at 4 °C. The F_1 segregating mapping population (code: 05-150) was obtained by crossing resistant parent A54750069-1 with susceptible plant 944750001-2. Population 07-407 containing *Rpi-dlc2* and *Rpi-dlc1* was obtained by crossing the resistant individual number 19 from the population 05-150 with the resistant individual number 50 from population 05-346 (Golas et al. 2010a).

Accessions and derived populations were cultivated at the Radboud University Experimental Garden. For germination, seeds were sown on moist soil, sprayed with GA_3 (Duchefa, The Netherlands) and placed in a 30 °C chamber. To regenerate plants from collected cuttings, parts of stem containing a node bud were placed for a period of 4 days in moist soil and covered with plastic foil to increase humidity. Ten-day-old seedlings and rooted cuttings were transplanted to one litre pots filled with a standard soil mixture. Plants were grown in the glasshouse under long day conditions (16 h light/8 h dark) with supplementary light of high-pressure sodium lamps (SON-T 600 W) and were regularly fertilized with 2 g/L standard plant fertilizer (Kristallon Blauw, Yara Benelux B.V. Vlaardingen). During winter, plants were kept in a cold glasshouse.

Detached leaf assays (DLAs)

DLAs were performed as described in Vleeshouwers et al. (1999), using *P. infestans* isolate Ipo655-2A races 1–11 (Flier et al. 2003b) collected in The Netherlands. Fresh sporangia were produced in a weekly cycle on detached leaves of the susceptible potato cultivar Bintje. Trays with leaves put into water-soaked florist foam were kept in closed plastic boxes for a period of 1 week in a climate chamber set at 18 °C (16 h light/8 h dark). A fresh suspension of zoospores was produced by rinsing around five

Table 1 Accessions of *S. dulcamara* selected for open-field experiment after the initial DLA screening

Accession code	Country of origin	Resistance screening	
		DLA[a]	Field[a]
A44750147-3	Poland	9	9
A54750069-1	Great Britain	9	8.75
A54750002-1	n.d.	8	8.75
A44750151-3	Poland	8	8.5
A54750003-3	n.d.	7	8.25
A44750149-3	Poland	7	8
A44750151-4	Poland	9	8
A44750149-4	Poland	8	7.5
A44750147-1	Poland	8	7.5
A54750007-3	The Netherlands	6	7.25
A44750081-3	n.d.	6	7
A54750003-1	n.d.	7	7
A54750007-1	The Netherlands	9	7
A54750066-1	Great Britain	7	6.75
A54750068-1	Great Britain	7	6.75
A54750009-4	The Netherlands	8	6.25
974750113-5	n.d.	7	6.25
A54750009-1	The Netherlands	6	5.75
A54750008-2	The Netherlands	6	5.5
A54750067-1	Great Britain	6	5.5
924750194-2	n.d.	6	5.25
974750113-4	n.d.	6	5.25
A54750005-2	The Netherlands	8	5.25
A54750008-4	The Netherlands	7	5.25
A44750090-3	n.d.	6	5
A44750182-4	n.d.	5	5
924750194-1	n.d.	6	4.75
A44750081-1	n.d.	7	4.5
A44750182-1	n.d.	7	4.5
A54750002-5	n.d.	5	4.25
A54750005-1	The Netherlands	7	4
A24750105-4	n.d.	7	3.5
A44750090-4	n.d.	5	3.25
A24750105-3	n.d.	5	2.5
944750001-2	Great Britain	1	1

n.d. not determined

[a] DLA and field resistance scores range from 1 (the most susceptible) to 9 (the most resistant response)

leaflets in approximately 200 mL tap water with a few drops of raw potato tuber sap added to it. After incubation of the sporangial suspension at 4 °C to induce zoospore release, inoculation was carried by applying two 10 µL drops of suspension to the adaxial side of the leaves. Infection severity was evaluated 7 days after inoculation. A plant was considered susceptible when leaves showed clear sporulation (resistance score 1) or sporulation was less intense (score ranging from 2 to 5) or only visible under stereoscopic microscope (resistance score 6). A plant was considered resistant when no sporulation was seen on developed necrotic tissue (score 7), if the necrotic reaction was strong (score 8), or only restricted to the site of inoculation (score 9).

Field trials

For each experiment, approximately 8-week-old plants were vegetatively propagated in April. A minimum of nine rooted cuttings were obtained per individual and potted in the second half of May. Around the 22nd of June 2005, 2006, 2007 and 2008, three plant plots of three plants each were planted on an experimental field near Marknesse (The Netherlands), as part of a much larger trial, where potato breeding material was tested for late blight resistance. At the end of July, spray inoculation was carried out with a suspension of *P. infestans* complex A2 isolate Ipo82001, races 1–7, 10 and 11 (Flier et al. 2003b). Observations on disease development were carried out in weekly intervals from mid of August till end of September. Four types of data were recorded: (1) an estimate of the amount of green and healthy tissue present on a plant, (2) an estimate of the severity of yellowed and dropped leaves, (3) the sporulation intensity, and (4) a total impression of plant fitness. A score on a scale from 1 (the most susceptible/unhealthy plant) to 9 (the most resistant/healthy plant) was assigned to each individual for each of the four parameters. Field resistance of a given individual was calculated as the average of all scores of all four parameters obtained during the testing season.

AFLP analysis

Total genomic DNA was isolated from young leaves using the Wizard genomic DNA purification kit (Promega, USA), according to the protocol supplied by the manufacturer. A pestle was used to grind approximately 40 mg of fresh plant material in liquid nitrogen to a fine powder. The concentration of DNA was measured using a spectrophotometer (Pharmacia Biotech: GeneQuant II) and the quality of the DNA was checked by electrophoresis in a 1 % agarose gel (Eurogentec, Belgium) stained with EtBr.

Amplified fragment length polymorphism (AFLP®) analysis was performed according to Vos et al. (1995). Initial digestion of total genomic DNA was done using *Eco*RI and *Mse*I restriction enzymes (Fermentas, Germany). Pre-amplification and selective PCR were performed using thermocycler GeneAmp9600 (Perkin Elmer, USA). Visualization of selective PCR products was done by labelling *Eco*RI primers with radioactive gamma-^{33}P (MP Biomedicals, USA). Labelled selective PCR products were separated on a 5 % polyacrylamide gel (Duchefa, The

Netherlands), dried on paper and visualized by exposure to X-ray film (Kodak BIOMAX MR) for 48 h. Presence/absence of bands was scored manually.

The bulked-segregant analysis approach was used to obtain AFLP fragments co-segregating with resistance (Michelmore et al. 1991). Two bulks were constructed by combining equal amount of pre-amplification product of five, either resistant or susceptible individuals. Bulks and parental DNA samples were analysed using 128 *Mse*I/*Eco*RI AFLP primer combinations. Primer combinations that yielded an AFLP product only in the resistant parent and the resistant bulk, but not it in the susceptible parent and the susceptible bulk, were identified and applied to all the individuals of segregating populations. To convert linked AFLP markers into PCR-based markers, the fragments were excised from the gel, eluted in water and re-amplified using AFLP primers for pre-amplification step. Subsequently, the resulting PCR fragments were cloned into pGEM-T Easy (Promega, USA) and sequenced using the CEQ^{TM} DTCS Quick Start Kit (Beckman Coulter 8000^{TM}). Based on the obtained sequence, primers were designed and tested for amplification. In case the primer pair itself was not allele specific, polymorphisms were searched within the amplified fragments by digestion of PCR generated fragments using 12 tetra-cutter restriction enzymes (*Alu*I, *Hpa*II, *Rsa*I, *Dpn*II, *Mse*I, *Bsu*RI, *Hha*I *Nla*III, *Dde*I, *Hin*fI, *Hpy*CH4IV, *Taq*I). Digestions were carried out for at least 2 h and they were checked on 1.5 % agarose gels stained with EtBr.

Comparative next-generation mapping

Total RNA was isolated from leaves of the resistant and susceptible parents and also from bulked leaves of 26 resistant and 28 susceptible genotypes of population 05-150 using the RNeasy kit (Invitrogen), treated with DNAse and polyA^{+} purified. Duplex-specific-nuclease-normalized cDNA samples were prepared and sequenced on the HiSeq2000 platform by Fasteris SA (Geneva, Switzerland), using manufacturer's protocols. For SNP calling, sequence reads were first trimmed and mapped to an existing *S. dulcamara* transcriptome assembly (unpublished results) using CLC Genomics Workbench v4.7.1 (CLC Bio, Aarhus, Denmark). For trimming, low-quality sequence (with limit 0.05) and ambiguous nucleotides (if longer than two nucleotides) were removed and reads shorter than 50 bases were discarded. For read mapping minimal read coverage was set at 90 % and minimal alignment identity at 90 %. Reads that could be mapped to multiple locations with the same score (repeats) were placed randomly to one of these locations. SNP calling was done using an upgraded version of QualitySNP (Tang et al. 2006; H. Nijveen, Wageningen University, The Netherlands, unpublished). To determine valid SNPs minimum similarity score per polymorphic site was set at 0.75 and minimum similarity score of all polymorphic sites at 0.8, INDEL SNPs were marked as low-quality and removal of low-quality at sequence ends was disabled. SNPs were taken as heterozygous in the resistant parent when coverage was more than 10 reads and frequency of each allele was at least 20 %. SNPs were taken as homozygous in the susceptible parent when coverage was more than 10 reads and the number of alternative allele reads was no more than 1.

Segregation was determined only for SNPs with a coverage of more than 20 reads in each of the bulks. The genetic distance of a SNP to the putative resistance locus was estimated from segregation of the minor allele (i.e. the resistant-parent-specific allele) in the bulk populations, using the fraction (Fr) of this minor allele in each of the bulks in the equation $Fr_{min}/(Fr_{max} + Fr_{min})$, where Fr_{min} is the lowest of the fractions of the minor allele in the two bulks, and Fr_{max} is the highest of the fractions of the minor allele in the two bulks. The running averages of the genetic distances of three serial SNPs were plotted against their putative position on the tomato genetic map. To this end, a BLAST-based analysis was performed to identify probable orthologous pairs as reciprocal best hits between bittersweet and tomato and bittersweet and potato, respectively. BLAST hits with bit scores lower than 100 were filtered out. In addition, BLAST searches were performed to identify near identical paralogs within the reference species tomato and potato. In case the bit score of a paralogous pair was higher than the score of the corresponding orthologous pair, the tomato/potato transcript was regarded as having in-paralogs (i.e. paralogs arising from duplication after speciation) and therefore the record was discarded from the analysis. The position of tomato genes on the genetic map was estimated through the identification of the closest 5′ marker from the tomato-EXPEN 2000 map available at the SGN ftp site (Mueller et al. 2005; ftp://ftp.solgenomics.net/maps_and_markers/Tomato/Tomato-EXPEN-2000.v1.txt). Physical positions were retrieved from a gff3 file that contains alignments of marker sequences to the tomato pseudo-molecules (http://solgenomics.net/itag/release/2.3/list_files/ITAG2.3_sgn_data.gff3).

PCR genotyping

Four SNPs (catalogued as SD markers) were selected in bittersweet contigs that flanked the linkage disequilibrium peaks based on synteny with tomato. All four SNPs were heterozygous in the resistant parent and homozygous in the susceptible parent, using the criteria described above. Ninety-one individuals of population 05-150 were genotyped for these SNPs using KASPar assays (KBioscience, Hoddesdon, UK). Primers were designed by KBioscience

and assays were performed according to the manufacturer's protocol on a Fluidigm EP1 system (Fluidigm Corporation, San Francisco, CA). *Rpi-dlc2* was mapped using data from a phenotyped subset of 26 resistant and 28 susceptible individuals of population 05-150.

Results

Identification of *S. dulcamara* plants sensitive or resistant to *P. infestans*

To identify *S. dulcamara* plants that were either sensitive or resistant to *P. infestans*, 19 accessions (15 of which were represented by five individuals and four by a single individual) were screened by DLAs. Plant number 2 from accession 944750001 (Golas et al. 2010a), known to be susceptible to the pathogen, was used as a control. The DLAs revealed a wide range of responses to the inoculation, with considerable variation in resistance even being present within a single accession. In four plants, from four different accessions, lesions were limited to the inoculation site (scored as 9, i.e. most resistant), whereas the susceptible control plant showed clear sporulation (scored as 1, i.e. most susceptible). From each accession, if available, the two most resistant individuals were identified and tested in an open-field experiment in 2005. As in case of the DLAs, the field experiment revealed a range of responses to *P. infestans* inoculations. Two of the individuals that showed the highest resistance in DLAs (A44750147-3 and A54750069-1) also proved to be the most resistant individuals in the field experiments, with resistance scores of 9 and 8.75, respectively (Table 1). The susceptible control plant 944750001-2 had a resistance score of 1, the lowest score for all the tested genotypes, and did not survive the infection. Altogether, there was a good correlation between the DLA results and open-field experiments (Table 1; Fig. 1).

Generation of a population segregating for *P. infestans* resistance

To be able to genetically map the *Rpi* gene, the highly resistant plants A44750147-3 and A54750069-1 were crossed with the susceptible plant 944750001-2. Two populations were obtained and screened using DLAs. In case of population 05-150, containing 63 individuals, a reproducible 1:1 segregation with 29 resistant, 28 susceptible and 6 undetermined genotypes ($\chi^2 = 0.57$; $P = 3.84$) was obtained. Subsequently, this population was tested under field conditions in 2006 and 2007. Parent plants were included as controls and showed the expected phenotype. The resistant parent remained largely free of disease

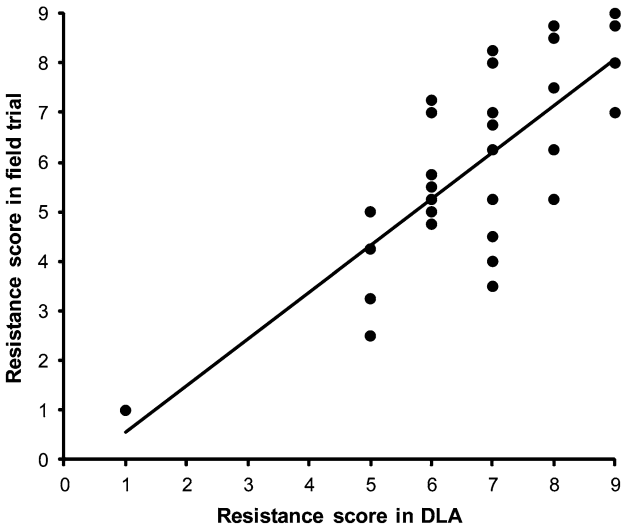

Fig. 1 Correlation between resistance scores of *S. dulcamara* accessions obtained in the open field trial of 2005 and detached leaf assays ($R^2 = 0.76$; $n = 35$; $P < 0.001$)

(scored as 8) and the susceptible parent developed intensive symptoms (scored as 3). All individuals of the offspring population showed higher resistance than the susceptible parent, but again there was a clear segregation into two distinct groups (Fig. 2). Phenotypic data for the individuals were comparable to those obtained earlier in DLAs and the resistance level was also inferred for the six individuals previously tagged as undetermined.

Identification of markers linked to *Rpi-dlc2* using AFLP analysis

As a first step towards mapping the resistance locus in population 05-150, the presence of *Rpi-dlc1* was excluded by analysing the linked markers GP41, GP101, TG591A-L and T0521 (Golas et al. 2010a). The TG591A-L marker revealed to be polymorphic between the parents when digested with restriction enzyme *Bsu*RI. Subsequent analyses on the individuals of 05-150 population showed that there was no correlation between the molecular marker and the resistance level, thereby excluding *Rpi-dlc1* as the gene responsible for the segregating resistance.

Population 05-150 was then used for AFLP analyses, yielding co-segregating fragments at 4.7 cM (eAACm CTG), 5 cM (eACCmCGC, eAAGmCTG) and at 16 cM (eAACmCCA, eAACmCAC) distance from the locus that was designated *Rpi-dlc2*. Two AFLP markers, generated by eAACmCTG (139 bp) and eAACmCCA (527 bp) were successfully converted into PCR-based markers (Table 2), whereas the remaining ones could not be converted because of failure in the amplification or in the identification of polymorphism useful for mapping experiments. Genotyping of the individuals of the 05-150 population showed that both

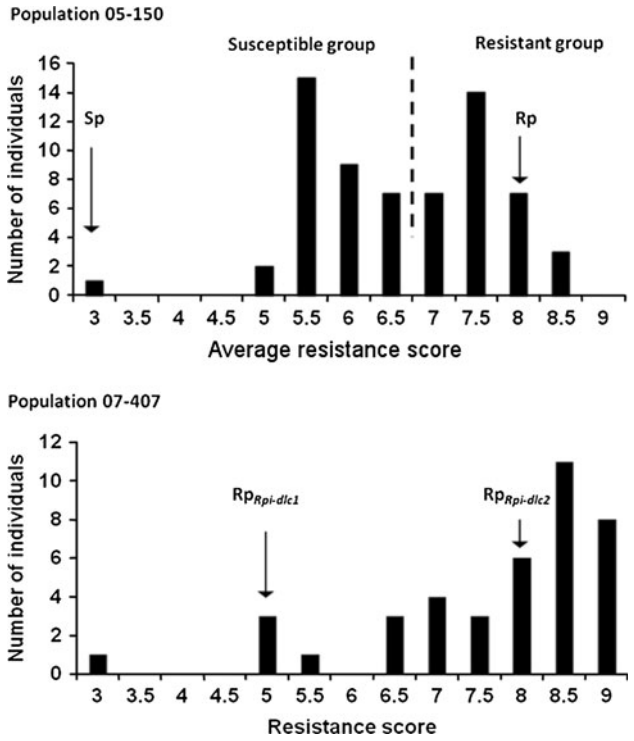

Fig. 2 Distribution of the phenotypes in F_1 populations 05-150 (containing 63 offspring) and 07-407 (containing 37 plants). The resistance score of the parents of each population is indicated. *Sp* susceptible parent, *Rp* resistant parent. For population 05-150, the average resistance score was determined from data of 2006 and 2007

PCR markers behaved identically to their corresponding AFLP markers, thereby confirming the correct identity of the amplified DNA fragment. The marker sequences were compared with the potato (v. 3_2.1.10) and tomato (v. 2.40) genomes, but no sequences with high similarity were identified. An attempt was made to position the markers on the genetic map of potato using the SHxRH potato mapping population (Van Os et al. 2006) and tomato insertion lines (Finkers et al. 2007), but apparent lack of sequence conservation between bittersweet and *S. tuberosum* and *S. lycopersicum* prevented the generation of PCR fragments suitable for such work.

Comparative mapping of *Rpi-dlc2* using next-generation sequencing

As an alternative approach to identify markers linked to the *Rpi-dlc2* locus, we employed next-generation sequencing of cDNAs of the parental clones and the resistant and susceptible bulks of population 05-150. First, all sequence information was used to assemble contigs, expected to represent the (partial) *S. dulcamara* transcriptome (unpublished results). Sequence information from the parents was then used to identify 4,844 SNPs present in a heterozygous state in the resistant parent and in a homozygous state in the susceptible parent (see "Materials and methods" for criteria). The genetic distance between these SNPs and the *Rpi-dlc2* locus was calculated by considering the segregation of the resistant-parent-specific allele in the resistant and susceptible bulks, which could be done reliably for 4,057 SNPs. Because of the extensive synteny between *Solanum* species (Fulton et al. 2002), the markers were subsequently positioned on the tomato genome by identifying the putative tomato ortholog of each *S. dulcamara* contig, which was successful in 3,575 cases. Fig. 3a shows that most of the markers that are in strong linkage disequilibrium are present on tomato chromosome 10, with two distinct peaks, one at ~30 cM and one at ~80 cM (Fig. 3b). To confirm these findings, we used sequence information to develop four PCR markers for *S. dulcamara*, flanking each of the two peaks on tomato chromosome 10. Again, these markers were selected to be heterozygous in the resistant parent and homozygous in the susceptible parent. Genotyping of the individuals of population 05-150 revealed the presence of an inversion of the second half of *S. dulcamara* chromosome 10 compared with tomato chromosome 10, with the breaking point between markers SD75 and SD76. Furthermore, *Rpi-dlc2* was mapped between markers SD79 and SD80, at a distance of 7.9 and 10.2 cM, respectively. Sequence comparison with potato genome pseudo-molecules (i.e. putative chromosomes) showed that SD79 is located between markers TG403 and TG63, which define the region of the *Rpi-ber2* locus and that SD80 is located between markers TG63 and CT240, which flank the *Rpi-ber/ber1* locus (Fig. 3c).

Phenotypic and molecular evaluation of a population containing *Rpi-dlc2* and *Rpi-dlc1*

To compare the effectiveness of *Rpi-dlc2* and *Rpi-dlc1* and evaluate the effect of combining the two R-genes, population 07-407 containing 37 individuals was tested for *P. infestance* resistance in DLAs and under field conditions in year 2008 (Fig. 2). The same individuals were genotyped with two sets of markers flanking either *Rpi-dlc2* or *Rpi-dlc1* (Table 3). In case one of the markers could not be determined (4 occasions) or recombination was observed between the flanking markers (3 occasions), the individuals were excluded from the analysis. Comparison of the phenotypic and molecular data of the remaining 30 individuals showed that all plants with the highest resistance score (>8) carried both *Rpi-dlc2* and *Rpi-dlc1*, or only *Rpi-dlc2*. A second group of resistant plants (score 7–8) harboured either *Rpi-dlc2* or *Rpi-dlc1*, with the exception of one plant that did not contain either of the two. Slightly susceptible plants (score 6–7) had either *Rpi-dlc1* or none of the two R-genes, while none of susceptible plants (score <6) contained *Rpi-dlc2* or *-1* (Fig. 4).

Table 2 Overview of the AFLP primer combinations generating co-segregating markers in population 05-150

AFLP primer combination	Primer sequences 5′ → 3′	Annealing temp./extension time	Restriction enzyme digestion
eAACmCTG	F: CTCCACTTGCGCTTCAAATC	55 °C/60 s	Allele specific
	R: GATTCAGATTTTCATAGGGT		
eAACmCCA	F: ATGGTTTATGTCATTATCATTG	50 °C/60 s	*Mse*I
	R: AGGAAATGGAATAATAGATACC		

Discussion

The goal of this study was to identify new genetic factors responsible for the resistance against *P. infestans* observed in *S. dulcamara* accessions (Golas et al. 2010b). Results published by Golas et al. (2010a), indicated the possibility that *Rpi* gene(s) other than *Rpi-dlc1* might be present in *S. dulcamara.* Indeed, an individual (A54750069-1) with a high level of resistance was identified and used to generate a population that segregated for resistance both in DLA and in field (Fig. 2). Independent segregation of the resistant phenotype and the molecular marker TG591A-L/*Bsu*RI linked to *Rpi-dlc1* pointed towards the presence of another gene in this population. Although the resistance level of the individuals of the population under field conditions was skewed towards that of the resistant parent, a binomial distribution could be observed that indicated the presence of a major gene contributing to resistance (Fig. 2). In this

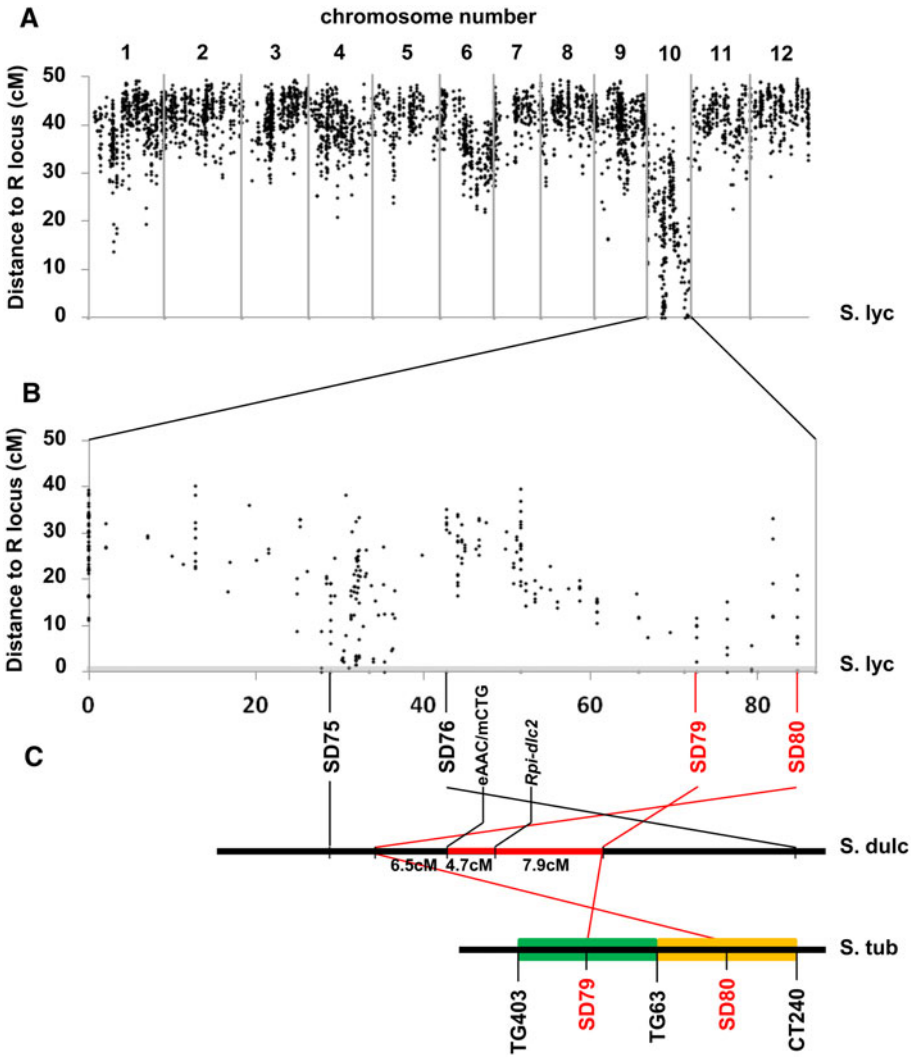

Fig. 3 Comparative mapping of *Rpi-dlc2*. **a** Genetic distances between *S. dulcamara* transcripts and the putative R locus, based on segregation of SNPs between bulked resistant and susceptible offspring, using comparative mapping against the tomato (*S. lyc*) genetic map. **b** A zoom-in on tomato chromosome 10 showing the genetic positions of the *S. dulcamara* markers SD75, -76, -79 and -80. **c** Schematic representation of the corresponding genetic regions in *S. dulcamara* (*S. dulc*) and *S. tuberosum* (*S. tub*), showing the position of *Rpi-dlc2* and an additional AFLP marker on the *S. dulcamara* map and the position of the *Rpi-ber/ber1* (*light grey bar*) and *Rpi-ber2* locus (*dark grey bar*) and their flanking markers on the potato genetic map

Table 3 Individuals of population 07-407 with corresponding resistance level and marker profiles

Individual	Resistance score	Marker profile			
		Rpi-dlc1		*Rpi-dlc2*	
		GP41/*Alu*I	GP101*Alu*I	eAAC/mCTG	eAAC/mCCA
1	6.5	1	1	0	0
2	8.5	0	0	1	1
3	8.5	1	1	1	1
4	8.25	1	1	1	1
5	8.4	1	1	1	1
6	8.8	1	1	1	1
7	8.45	1	1	1	1
8	7.54	0	0	1	1
9	7.81	1	1	0	0
10	5.2	0	0	0	0
11	9	0	0	1	1
12	5.1	0	0	0	0
13	6.9	0	0	0	0
14	6.8	1	1	0	0
15	8.6	1	1	1	1
16	6.1	1	1	0	0
17	7.3	0	0	0	0
18	6.81	1	1	0	0
19	8.1	1	1	1	1
20	7.1	1	1	0	0
21	8.8	1	1	1	1
22	6.1	0	0	0	0
23	8	0	0	1	1
24	8	0	0	1	1
25	8.1	0	0	1	1
26	8.54	1	1	1	1
27	8.72	1	1	1	1
28	7	1	1	0	0
29	8	0	0	1	1
30	7.63	1	1	0	0

1 marker present, 0 marker absent

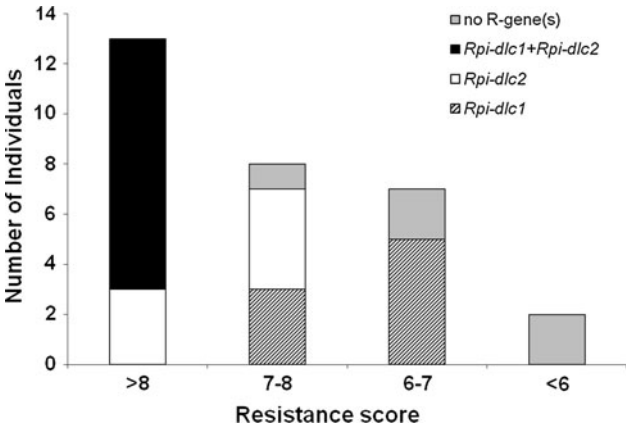

Fig. 4 Distribution of resistance scores of 30 population 07-407 offspring clones in relation to presence or absence of resistance genes *Rpi-dlc1* and *Rpi-dlc2* as inferred from gene associated markers. Resistance scores ranged from 1 to 9, where 1 means susceptibility to late blight and 9 immunity to late blight. By different shading, the number of individuals assigned to each phenotypic class is sub-grouped according to the presence or absence of either gene

paper we describe the identification, field evaluation and mapping of a novel gene, named *Rpi-dlc2,* which confers resistance against late blight.

The AFLP technique is widely used for genetic mapping of traits in species for which limited genome sequence information is available. Indeed, this technique was successfully used to map the *P. infestans* resistance gene, *Rpi-dlc1*, in *S. dulcamara* (Golas et al. 2010a). Initial mapping of *Rpi-dlc2* using AFLP also resulted in the identification of markers linked to the resistant phenotype. However, low sequence conservation between the AFLP fragments and the tomato and potato genomes hampered comparative genetic positioning of the gene. Deep sequencing of *S. dulcamara* cDNA successfully overcame this bottleneck by enabling characterization of thousands of cDNAs and the identification of several thousands of reliable SNPs within them. Because cross-species sequence conservation is much higher for the protein-coding regions, as present in the cDNAs, than for most of the non-coding sequence of the genome, a large fraction of the *S. dulcamara* cDNAs, and thus the SNPs within them, could be positioned on the tomato genome with high confidence. Linkage of the SNPs to the phenotype could then be determined in a bulked-segregant analysis using genotyping-by-sequencing, also known as next-generation mapping (Lister et al. 2009; Austin et al. 2011). Taken together, "comparative next-generation mapping" is a very powerful approach in cases where there is no a priori sequence information of the species of interest, but the genome sequence of a related species is available. In addition, in the process many SNPs are identified that are useful for further PCR-based fine mapping.

Based on this information and on the availability of tomato and potato sequence as a reference, *Rpi-dlc2* could be positioned on the equivalent of chromosome 10 of *S. dulcamara* in an R-gene cluster known to harbour resistance against late blight derived from *S. berthaultii* (Rauscher et al. 2006; Park et al. 2009). Besides *Rpi-ber* genes, this cluster contains also the major tomato late blight resistance gene *Ph-2* (Moreau et al. 1998) and resistance to *Globodera rostochiensis*, *Gro1.2* (Leister et al. 1996). Because this position is different from that of *Rpi-dlc1*, which has been mapped on chromosome 9 (Golas et al. 2010a), we conclude that at least two resistance genes are present in *S. dulcamara* that contribute to the protection of this plant species against late blight.

DLAs and field experiments showed that *Rpi-dlc2* stops *P. infestans* at an early stage of infection and the pathogen is not able to close the life cycle (data not shown). Furthermore, our resistance assays revealed a strong cell death-associated defence reaction known as hypersensitive response, like triggered by resistance genes *Rpi-ber* and *Rpi-blb1*, from the American *Solanum* species *S. berthaultii* and *S. bulbocastanum,* respectively (Ewing et al. 2000; Van der Vossen et al. 2003). By contrast, the previously identified *Rpi-dlc1* has weak R-gene properties, similarly to the *Rpi-mcd1* described by Tan et al. 2008. Crossing of two parental clones containing *Rpi-dlc1* and *Rpi-dlc2,* respectively, resulted in population 07-407. By marker-assisted selection, groups of offspring from 07-407 were created that contained neither of these genes, *Rpi-dlc1* or *Rpi-dlc2* alone or a combination of both. Results from the screening of these groups under field conditions showed that *Rpi-dlc2* alone gave a stronger resistance than *Rpi-dlc1* alone and that all individuals containing both *Rpi-dlc2* and *Rpi-dlc1* fell in the class of individuals with the highest resistance level. Similarly, Tan et al. (2010) showed for potato that the combination of the resistance genes *Rpi-mcd1* and *Rpi-ber* resulted in a significant reduction of the infection, compared with plants carrying only one of the two genes.

It is questionable now if *Rpi-dlc2* and *Rpi-dlc1* are part of much larger array of R-genes employed by *S. dulcamara* against *P. infestans*, like in the case of, e.g. *S. demissum* where up to 11 R-genes were identified (Gebhardt and Valkonen 2001). Indeed, the bias of population 05-150 towards the resistance of the R parent (Fig. 2) may point at such additional R-genes. Thus, potentially, *S. duclamara* is a rich source of new R-genes that might supplement *Rpi*-genes from New World Solanaceae. Identifying the effectors from *P. infestans* that are functional to *Rpi-dlc1* and *Rpi-dlc2* would allow studying their virulence spectrum and elucidating whether the *Rpi*-genes of *S. dulcamara* may complement the already known *Rpi*-genes from tuber bearing Solanaceae (Vleeshouwers et al. 2008).

It is widely perceived that newly introduced R-genes against *P. infestans* in crop plants such as tomato and potato only provide full resistance for a limited period of time, until strains of the pathogen evolve that render it capable of avoiding recognition by the host (Nowicki et al. 2012). Natural late blight infections of *S. dulcamara* are only rarely observed, however, and if so, the disease progresses only to a limited extend (Cooke et al. 2002; Flier et al. 2003a). Further investigation of bittersweet R-genes may provide new insights into the role that *Rpi-dlc2* and *Rpi-dlc1* play in the overall strategy *S. dulcamara* employs to defend itself against *P. infestans* in case of intense disease pressure during the potato growing season (Golas et al. 2010b). In addition, the molecular mechanisms underlying the observed more durable resistance in *S. dulcamara,* compared with the potato/late blight pathosystem need to be elucidated.

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