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Allele mining in the gene pool of wild *Solanum* species for homologues of late blight resistance gene *RB/Rpi-blb1*

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Abstract

A coiled coil-nucleotide binding site-leucine-rich repeat gene *RB/Rpi-blb1* isolated from *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* and is currently employed in potato breeding for durable late blight (LB) resistance. *RB* homologues were reported in several *Solanum* species; some of them retained defence function. Here, we report additional evidence on *RB*-like sequences in 21 *Solanum* species of the section *Petota*. The panel of *Solanum* species was screened with three *RB*-related PCR markers. *RB*-like sequences were found in every tested *Solanum* accession, suggesting universal distribution of *RB* structural homologues among *Solanum* genomes, while locus-specific RB-629 was found only in 15 species. Phylogenetic analysis of RB-629 sequences suggested a highly conservative pattern of polymorphisms that was neither species- nor series-specific. Apparently, duplication and evolution of *RB*-like loci preceded *Solanum* speciation. Marker presence and particular haplotypes were not immediately associated with high LB resistance.

Keywords: late blight resistance; *Phytophthora infestans*; potato; *R* genes; *Solanum spp.*

Introduction

Late blight (LB, pathogen *Phytophthora infestans*) resistance mediated by the *R* genes is one of the integral elements of plant immune system (Dangl and Jones, 2001). Cultivated potato (*Solanum tuberosum*) lacks *R* genes active against *P. infestans*, apparently due to the vegetative propagation excluding natural selection for functional *R* loci under the recurrent pathogen attacks. On the other hand, wild *Solanum* species inhabiting regions with the most diverse populations of *P. infestans* acquired numerous *R* loci functional against LB and are essential genetic resources for potato breeding.

A set of 11 *R* genes was identified in the Mexican species *S. demissum* and introgressed into potato varieties, but resistance was supposedly defeated in the field by rapidly evolving *P. infestans* races (Fry, 2008). Several genes of LB resistance were mapped on the linkage groups of various wild *Solanum* species (Hein *et al.*, 2009). A cluster of four resistance gene analogues (*RGA*s) located on chromosome 8 of *S. bulbocastanum* was cloned and *RGA2* (*Rpi-blb1/RB*) conferred resistance to LB in both transient and stable expression systems (Song *et al.*, 2003; van der Vossen *et al.*, 2003). Potato transformation with *RB* homologues isolated from *S. bulbocastanum* (*Rpi-bt1*), *S. stoloniferum* (*sensu* Spooner *et al.*, 2004; *Rpi-sto1*, *Rpi-pta1*) and *S. verrucosum* (RB^{ver}) confirmed specificity of these genes against broad spectrum of *P. infestans* races (Liu and Halterman, 2006; Vleeshouwers *et al.*, 2008; Oosumi *et al.*, 2009). However, recently, *P. infestans* races lacking Avr effectors compatible with RB ligand and thus virulent on potato plants transformed with *RB* have been identified (Champouret *et al.*, 2009; Förch *et al.*, 2010; Halterman

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et al., 2010). Pyramiding broad-spectrum resistance genes from various sources with different specificity to pathogen races in potato genome is probably a more effective approach to durable LB resistance of potato cultivars (Tan *et al.*, 2010).

In the present study, we followed an effective and efficient allele mining approach (Wang *et al.*, 2008) to analyse distribution and diversity of *RB*-like candidate resistance genes in germplasm of the wild *Solanum* species section *Petota*. Conservative patterns of polymorphisms were specific for paralogous *RB*-like loci rather than for *Solanum* species, thus suggesting that *RB* homologues duplicated and diverged before *Solanum* speciation.

Materials and methods

Genomic DNA was isolated from 139 accessions representing 21 wild *Solanum* species (Supplementary Table S1, available online only at http://journals.cambridge.org), using AxyPrep™ Multisource Genomic DNA Miniprep Kit. To amplify *RB*-like homologues, we designed universal RB-1223 and locus-specific RB-629 PCR primers (Table 1 and Supplementary Fig. S1, available online only at http://journals.cambridge.org) and optimised them using OligoCalc (Kibbe, 2007). We also modified the allele-specific PCR primers 1 and 1′ recognising a functional allele of *bulbocastanum RB* (Colton *et al.*, 2006; RB-226) to increase reaction specificity. The amplification reactions contained 1 μl of 10 × PCR buffer, 100–150 ng of genomic DNA, 1 μl 2.5 mM dNTP, 10 pmol each of two primers, 1 U of either *Pfu* (cloning; Fermentas) or *Taq* (screening; Syntol) DNA polymerase and sterile water to a volume of 10 μl, and were run in an MJ PTC-200 thermocycler (Bio-Rad). PCR products were separated by electrophoresis in 1.5% (w/v) agarose and stained with ethidium bromide. Amplified fragments were cloned using InsTAclone™ and CloneJET™ PCR Cloning Kits (Fermentas) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 3730 DNA Analyzer (Applied Biosystems). DNA sequences were analysed using BLAST 2.2.23 (Altschul *et al.*, 1990), Lasergene 6.0 (DNAStar) and ExPASy Translate tool (http://www.expasy.org). Cluster analysis was performed using Maximum likelihood algorithm with 1000 bootstrap replicates implemented in Phylip 3.69 (Felsenstein, 1989). LB resistance was assessed using a modified detached leaf assay (Filippov *et al.*, 2004; Kuznetsova and Rogozina, unpublished data).

Results and discussion

Based on their structural polymorphisms, the functionally active *RB*-like loci can be arranged into three distinct groups: *RB*-group (*RB*, *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1*), RB^{ver}-group and *Rpi-bt1*-group. It is noteworthy that exonic regions of *RB*-like loci retained over 90% homology, while introns diverged dramatically after duplication of the *RB*-like loci. Apparently, these groups represent orthologous loci which emerged from different *RB*-like paralogues duplicated in ancient *Solanum* species and independently acquired defence function against LB under the selective pressure of the pathogen invasion events after *Solanum* speciation.

In order to investigate the distribution of *RB*-like genes in the wild *Solanum* germplasm, three sequence characterised amplified region (SCAR) markers were designed: RB-1223 tagging all three groups of *RB*-like loci, RB-629 specific for *RB*-group only and allele-specific RB-226 (Supplementary Fig. S1, available online only at http://journals.cambridge.org). Marker RB-1223 was used to screen 22 accessions representing 13 species (*S. avilesii*, *S. bulbocastanum*, *S. ehrenbergii*, *S. demissum*, *S. hjertingii*, *S. hougasii*, *S. iopetalum*, *S. microdontum*, *S. pinnatisectum*, *S. polyadenium*, *S. polytrichon*, *S. stenophyllidium*, *S. stoloniferum* and *S. verrucosum*); this marker was universally present in every tested accession, suggesting ubiquitous distribution of the *RB*-like loci in *Solanum* genomes. RB-1223 was present in several copies (one to three discernable bands/accession) and greatly varied in size (~800–1300 bp). Sequencing experiments revealed that polymorphic bands in various *Solanum* species corresponded to paralogous and orthologous *RB*-like loci.

Table 1. PCR primers for amplification of the *RB*-related SCAR markers

Markers	Primers	PCR thermal profiles
RB-1223	F 5′-atggctgaagctttcattcaagttctg-3′ R 5′-caagtattgggaggactgaaaggt-3′	3 min at 94°C; 35 cycles 45 s at 94°C, 45 s at 65°C, 1 min 20 s at 72°C; 15 min at 72°C
RB-629	F 5′-gaatcaaattatccaccccaacttttaaat-3′ R 5′-caagtattgggaggactgaaaggt-3′	3 min at 94°C; 35 cycles 45 s at 94°C, 45 s at 65°C, 1 min 20 s at 72°C; 15 min at 72°C
RB-226	F 5′-cacgagtgcccttttctgac-3′ R 5′-ttcaattgtgttgcgcactag-3′	3 min at 94°C; 35 cycles 30 s at 94°C, 30 s at 60°C, 1 min at 72°C; 15 min at 72°C

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Observed variation in size was mainly due to the polymorphisms in introns (Pankin *et al.*, unpublished data).

The panel of the 134 accessions of 19 *Solanum* species was screened with RB-629 and RB-226 markers. RB-629 was present in 54% of accessions representing 15 species, whereas allele-specific RB-226 was found only in 7% of accessions from five species (Supplementary Table S1, available online only at http://journals.cambridge.org). Our data suggest wider distribution of *RB*-group loci in *Solanum* germplasm than reported earlier (Wang *et al.*, 2008; Lokossou *et al.*, 2010). RB-226 was also found both in resistant and susceptible *Solanum* accessions, including *S. bulbocastanum*, and therefore cannot be universally used to discern the active *RB* allele even in *S. bulbocastanum* accessions.

RB-629 was cloned from 16 accessions representing 12 *Solanum* species (Supplementary Table S1, available online only at http://journals.cambridge.org). Phylogenetic analysis of RB-629 sequences produced four distinct clusters: cluster 1 of *bulbocastanum*-like haplotypes, cluster 2 comprising pseudogenes except one *pinnatisectum* RB-629 (pnt2), cluster 3 specific for *S. polytrichon*

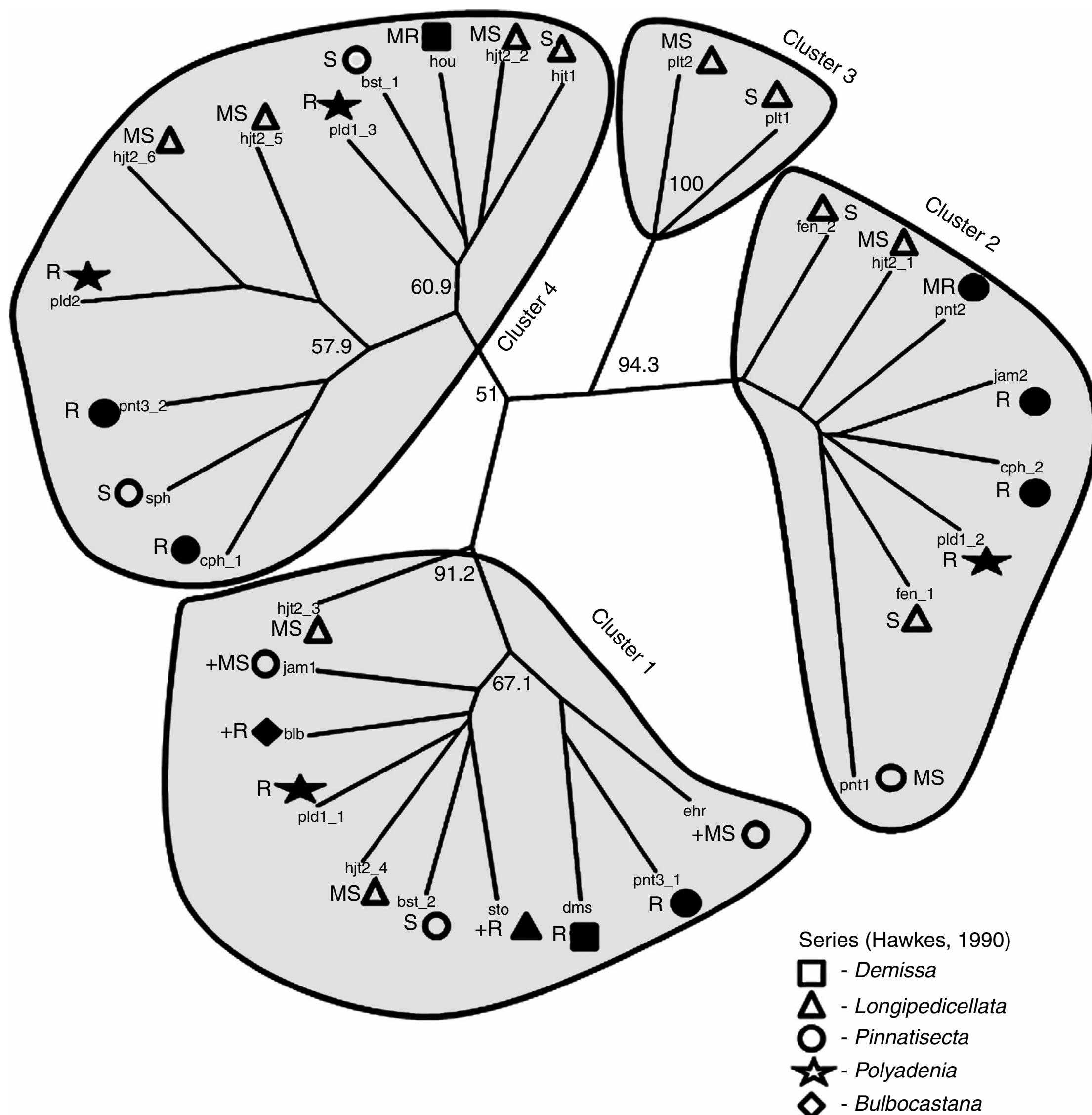

Fig. 1. Phylogenetic analysis (maximum likelihood) of the *RB* fragments (RB-629). +, presence of allele-specific RB-226. Resistance ranks are as follows: open symbols: S, susceptible, MS, moderately susceptible; closed symbols: MR, moderately resistant, R, resistant. Bootstrapping was performed with 1000 replicates, and values higher than 50% are shown at the nodes. Cluster 1, *Rpi-blb*/*RB*-like haplotypes; cluster 2, pseudogenes; cluster 3, *S. polytrichon*-specific haplotypes; cluster 4, other haplotypes. Sequence abbreviations with underscore tag are either allelic or homeologous variants of RB-629. For the list of abbreviations and sequences, refer to Supplementary Table S1 (available online only at http://journals.cambridge.org).

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and cluster 4 combining other *RB*-group sequences with open reading frame (Fig. 1). The described pattern of polymorphisms was neither species- nor series-specific; thus the observed diversity of *RB*-group loci emerged before *Solanum* speciation and probably is not linked to allopolyploidisation in *Solanum* species. Apparently, each cluster combines allelic variants of *RB* orthologues, whereas inter-cluster polymorphisms are indicative of different *RB* loci. Despite the defence function against LB unequivocally demonstrated in complementation experiments with *RB* genes, the presence and polymorphisms of *RB* sequences in various *Solanum* species were not immediately associated with higher LB resistance. Redundant copies of *RB*-like paralogues apparently serve as a backup pool essential to the adaptive evolution of *R* gene-related pathogen recognition when *Solanum* species respond to novel races of pathogen.

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