Searching among wild *Solanum* species for homologues of *RB/Rpi-blb1* gene conferring durable late blight resistance

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SUMMARY

Solanum bulbocastanum comprising a CC-NBS-LRR gene *RB/Rpi-blb1* confers broad-spectrum resistance to *Phytophthora infestans* and is currently employed in potato breeding for durable late blight (LB) resistance. Genomes of several *Solanum* species were reported to contain *RB* homologues with confirmed broad-spectrum defence function. With the discovery that novel *P. infestans* races break LB resistance mediated by the genes of broad-spectrum specificity, pyramiding several *RB*-like genes from various *Solanum* species in a single potato cultivar seems a promising approach to durable LB resistance. Here we report early evidence on *RB*-like sequences in the wide range of *Solanum* species 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 the marker RB-629 corresponding to the *RB* gene was found in 14 species. The phylogenetic analysis of RB-629 sequences suggested highly conserved 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

Phytophthora infestans, Solanum spp., late blight resistance, *R* genes, potato

INTRODUCTION

Late blight (LB) caused by *Phytophthora infestans* (Mont.) de Bary is still challenging potato fields around the globe. Disease resistance mediated by the *R* genes is one of the integral elements of the plant immune system. Products of *R* genes directly or indirectly recognise the cognate effector (Avr) which is introduced into the plant cell by the pathogen and induces the hypersensitive response (Dangl and Jones, 2001). Cultivated potato (*Solanum tuberosum* L.) lacks *R* genes active against *P. infestans,* primarily due to the practice of vegetative propagation that excludes natural selection for

functional *R* loci under recurrent pathogen attacks. In contrast, wild *Solanum* species inhabiting the regions with the most diverse populations of *P. infestans* acquired numerous *R* loci that are functional against LB and are indispensable genetic resources for potato introgression breeding.

The set of eleven *R* genes was identified in the Mexican species *S. demissum* and introgressed into potato varieties. However, such resistance was reportedly defeated in the field by rapidly evolving *P. infestans* races (Fry, 2008; Hein *et al.*, 2009). Nonetheless, the presence of some *demissum R* genes in potato cultivars was explicitly associated with high LB resistance indices (for more details see Khavkin *et al.* in this issue). Several QTLs and genes for LB resistance have been mapped on the linkage groups of various wild *Solanum* species (Hein *et al.*, 2009). A cluster of four resistance gene analogues (RGAs) located on chromosome 8 of *S. bulbocastanum* was cloned, and *RGA2* (*RB*/*Rpi-blb1*) was shown to confer LB resistance 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 a broad spectrum of *P. infestans* races (Liu and Halterman, 2006; Vleeshouwers *et al.*, 2008; Oosumi *et al.*, 2009). 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 *et al.*, 2010). From the breeding prospect, many wild *Solanum* species exhibiting high levels of LB resistance cannot be crossed with *S. tuberosum* by conventional breeding methodologies. Thus, cloning and functional characterisation of the genes underlying broad-spectrum LB resistance would promote immediate exploitation of wild *Solanum* germplasms in potato breeding. Pyramiding, by cisgenesis, in potato genome broad-spectrum *R* genes from various sources with different specificity to pathogen races and with additive effect is a promising approach to durable LB resistance of potato cultivars (Tan *et al.*, 2010).

In the present study, we employed an effective and efficient allele mining approach to demonstrate the universal distribution and diversity of *RB*-like candidate *R* genes within wild *Solanum* germplasm. We revealed conserved patterns of polymorphisms specific for paralogous *RB*-like loci rather than for *Solanum* species and tentatively suggest that *RB* homologues duplicated and diverged preceding *Solanum* speciation.

MATERIAL AND METHODS

Plant material and DNA isolation

Seeds of wild *Solanum* species were obtained from The Centre for Genetic Resources (CGN), the Netherlands, NRSP-6 Potato Genebank (PI), USA, and The Vavilov Institute of Plant Industry (VIR), Russia. Genomic DNA was isolated from individual plants of 134 accessions representing 18 wild *Solanum* species, section *Petota* (Table 1), by modified CTAB isolation (Doyle and Doyle, 1987) and AxyPrep™ Multisource Genomic DNA Miniprep Kit.

SCAR markers design, amplification and cloning

RB-like homologues were amplified from genomic DNA using universal sequence characterised amplified region (SCAR) markers RB-1223 tagging several *RB*-like loci and marker RB-629 specific for the *RB* gene. PCR primers were optimised using the Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools). Allele-specific PCR primers 1 and 1' recognising functional allele of *S. bulbocastanum RB* (Colton *et al.*, 2006; RB-226) were modified to increase reaction specificity. The amplification reactions contained 1 µl of 10x 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* DNA polymerase (Fermentas) for cloning) or *Taq* DNA polymerase (Syntol) for screening and sterile water to a volume of 10 µl and were run in an MJ PTC-200 thermocycler (Biorad). 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).

Phylogenetic analysis of RB-629 sequences

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, Neighbor-joining and Maximum parsimony algorithms implemented in Phylip 3.69 (Felsenstein, 1989).

LB resistance assays

LB resistance scores of individual *Solanum* plants were independently determined in the laboratory and field trials at the Institute of Phytopathology and at the Institute of Plant Industry (for the protocols see Rogozina *et al.*, this issue).

RESULTS AND DISCUSSION

Comparative analysis of *RB*/*Rpi-blb1* homologues exposed conserved structure of exonic regions (over 90% identity) and dramatically polymorphic introns supposedly diverged already after duplication of the *RB*-like loci.

Based on discovered polymorphisms, the functionally active *RB*-like loci can be provisionally arranged into three distinct groups: *RB*-group (*RB*, *Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1*), RB^{ver}-group and *Rpi-bt1*-group. Apparently, these groups represent orthologous loci, which emerged from the different *RB*-like paralogues duplicated in ancient *Solanum* genotypes and independently acquired defence function against LB under the selective pressure of the pathogen invasion events following *Solanum* speciation.

In order to investigate the distribution of *RB*-like genes in the wild *Solanum* germplasm, three SCAR markers were designed: RB-1223 tagging all three groups of *RB*-like loci, RB-629 specific for *RB*-group and allele-specific RB-226 (Fig. 1). Marker RB-1223 was used to screen 19 accessions representing 11 species (*S. bulbocastanum*, *S. cardiophyllum* ssp. *ehrenbergii*, *S. demissum*, *S. hjertingii*, *S. hougasii*, *S. iopetalum*, *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* homologues in *Solanum* genomes. The RB-1223 marker was present in several copies (1-3 copies per accession) and greatly varied in size (~800 to 1300 bp). Sequencing experiments showed that polymorphic bands of this marker in various *Solanum* accessions corresponded to paralogous *RB*-like loci. The observed variation in size was mainly due to the polymorphisms in the intron (Pankin *et al.*, unpublished data).

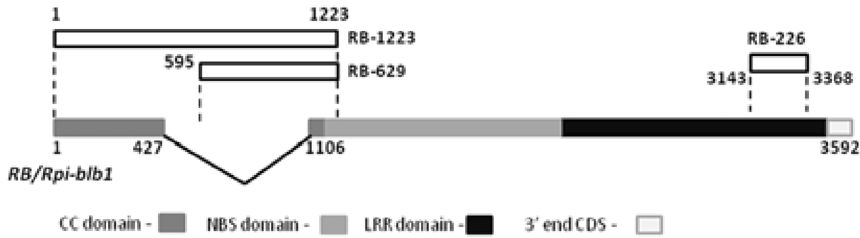

Figure 1. *Schematic structure of the RB/Rpi-blb1 gene and locations of SCAR markers. Numbers are nucleotide positions in base pairs (bp) in relation to the full-length RB/Rpi-blb1 gene sequence (AY426259, 3528 bp). Lines angled downwards indicate the position of 680-bp long intron.*

The panel of the 134 accessions of 18 *Solanum* species was screened with the locus-specific RB-629 and allele-specific RB-226 markers. RB-629 was present in 54% of accessions representing 14 species, whereas allele-specific RB-226 recognising characteristic 18-bp long indel was found only in 7% of accessions from five species (Table 1). Our data suggest much wider distribution of *RB*-group loci in *Solanum* germplasm than reported earlier (14 species vs. two species reported by Wang *et al.*, 2008 and four species reported by Lokossou *et al.*, 2010). In addition to *S. bulbocastanum*, *S. cardiophyllum* subsp. *cardiophyllum*, and *S. stoloniferum* reported by Lokossou *et al.* (2009), three more species *S. caridiophyllum* subsp. *ehrenbergii*, *S. jamesii* and *S. pinnatisectum* were found to contain marker RB-226. It is remarkable that we found RB-226 attributed to the functionally active *S. bulbocastanum RB* allele (Colton *et al.*, 2006) both in resistant and susceptible *Solanum* accessions including *S. bulbocastanum*. It follows that RB-226 cannot be universally used to discern the active *RB* allele even in *S. bulbocastanum* accessions.

Table 1. *Results of the screening of Solanum germplasm with RB-group specific SCAR markers RB-629 and RB-229.*

Specific amplification		*Solanum* accessions
RB-629	RB-226	
+	+	**blb** PI 243510, 275198, VIR 21266-432, 23174-510; **ehr** *VIR 24373-425 (ehr)*; **jam** *VIR 15203-349 (jam1)*; **pnt** VIR 19328-362; **sto** PI 365401, VIR 24263
+	-	**blb** PI 243506, 243508, 243509, 243512, VIR 21274-509-1, 21274-509-2, 23181-511; **bst** VIR 23201, 23201-344, *24197-345 (bst)*; **crd** VIR 4464, *16828-390 (cph)*, 23030-410; **dms** PI 161366, 175408, 230487, *VIR 15173 (dms)*, 23315-277; **ehr** VIR 21301, 23276, 24572-520-2; **fen** *VIR 23335-286 (fen)*, 23841, 24221; **hjt** PI 283103, *VIR 15194-291 (hjt2)*, *21409 (hjt1)*, 24223; **hou** *VIR 8818 (hou)*, 8818-278; **jam** PI 612455, *VIR 22619-351 (jam2)*, 24397, 24397-359; **pld** *VIR 23546-532 (pld2)*, *23553-334 (pld1)*; **pnt** PI 186553, 275234, VIR 19327, 21955, *21955-363 (pnt1)*, 24239, *24239-367 (pnt2)*, *24243-368 (pnt3)*, 24415, 24415-370; **pta** VIR 21547, 24417; **plt** VIR 16905, *23556-315 (plt1)*, 23561-316, 24298-318, 24410, 24462-321, *24463-322 (plt2)*; **sph** PI 320265, *VIR 24255-380 (sph)*; **sto** CGN 17606, PI 161178, 205510, 230490, 255533, 255534, 310964, 338621, VIR 3360, 3336-324, 18925, 19196-325, 21618-327, 23652, 23652-329, 24420-330
-	-	**bcp** VIR 2830-273; **blb** PI 255516, 275191, 275200, VIR 19981-431, 21274-509-3, 23181-511-1, 23181-511-2; **bst** VIR 20105-378, 24197; **crd** VIR 24375-426; **dms** PI 160221, 161167, 161176, 175404, 186552, 218047, 275211, VIR 15174, 18521-275; **ehr** PI 255520, VIR 18086, 18224, 18225, 23277, 23279-414, 24206-419, 24207-420, 24572-520-3; **fen** VIR 24218-288; **hjt** VIR 19276-293, 24387; **hou** PI 161727; **iop** PI 230459; **jam** VIR 15203, 23397-352, 23398-353, 23399-354; **pld** PI 275237, 275238; **plt** VIR 8815, 24463; **pnt** PI 275233, VIR 4213; **pta** VIR 8816, 16889; **trn** VIR 23936-381; **sto** CGN 17607, PI 558477; **ver** PI 365404, VIR 23015-555, 23760-556

RB-629 was cloned from 16 accessions representing 12 *Solanum* species (Table 1). The phylogenetic analysis of RB-629 sequences produced four distinct clusters: cluster 1 of *S. bulbocastanum*-like haplotypes; cluster 2 comprising pseudogenes, except one *pinnatisectum* RB-629 fragment (pnt2); cluster 3 specific for *S. polytrichon;* and cluster 4 combining other *RB*-group sequences with open reading frame (Fig. 1). Remarkably, the Maximum likelihood, Neighbor-joining and Minimum parsimony algorithms when applied to the same dataset produced congruent trees with high bootstrap values, thus suggesting high reliability of the revealed clustering.

Abbreviations: *Solanum* species (Hawkes *et al.*, 1994) - *S. brachycarpum* Correll (bcp), *S. brachistotrichum* (Bitter) Rydb. (bst), *S. bulbocastanum* Dunal (blb), *S. cardiophyllum* John Lindley (cph), *S. cardiophyllum* ssp. *ehrenbergii* Bitter (ehr), *S. demissum* Lindl. (dms), *S. fendleri* (fen), *S. hjertingii* Hawkes (hjt), *S. hougasii* Correll (hou), *S. iopetalum* (Bitter) Hawkes (iop), *S. jamesii* Bitter (jam), *S. papita* Rydb. (pta), *S. pinnatisectum* Dunal (pnt), *S. polyadenium* Greenm. (pld), *S. polytrichon* Rydb. (plt), *S. stenophyllidium* Bitt. (sph), *S. stoloniferum* Schltdl. (sto), *S. tarnii* Hawkes & Hjert. (trn), *S. verrucosum* Schtdl. (ver). Genbanks: CGN – The Centre for Genetic Resources, the Netherlands, PI - NRSP-6 Potato Genebank, USA and VIR – the Vavilov Institute of Plant Industry, Russia. Accession numbers with sequenced RB-629 marker and corresponding sequence names in parentheses as shown in Fig. 1 are italicised. Resistant accessions (R and MR) are highlighted in grey. Accessions without LB resistance scores are underlined.

The described pattern of polymorphisms was neither species- nor series-specific. Therefore, we suggest that the observed diversity of *RB*-group loci emerged before *Solanum* speciation. Apparently, each cluster combines allelic variants of *RB* orthologues whereas inter-cluster polymorphisms were indicative of different *RB* loci. Despite the defence function against LB unequivocally demonstrated in complementation experiments with *RB* genes (Song *et al.*, 2003; van der Vossen *et al.*, 2003; Vleeshouwers *et al.*, 2008), the presence and polymorphisms of *RB* sequences in various *Solanum* species was not immediately associated with higher LB resistance. Apparently, *RB*-like genes duplicated in *Solanum* genomes are of ancient origins (van der Vossen *et al.*, 2003). Defence function of *RB* orthologues could emerge after speciation independently in various *Solanum* species, under the selective pressure of the pathogen, as they spread over the Americas. Another model explaining abundance of non-functional *RB* loci in *Solanum* species is the loss of function of either *RB* genes due to the frame-shifting nucleotide mutations or any downstream elements involved in the signalling cascade of the defence response.
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 (for review see Hubert *et al.*, 2001).

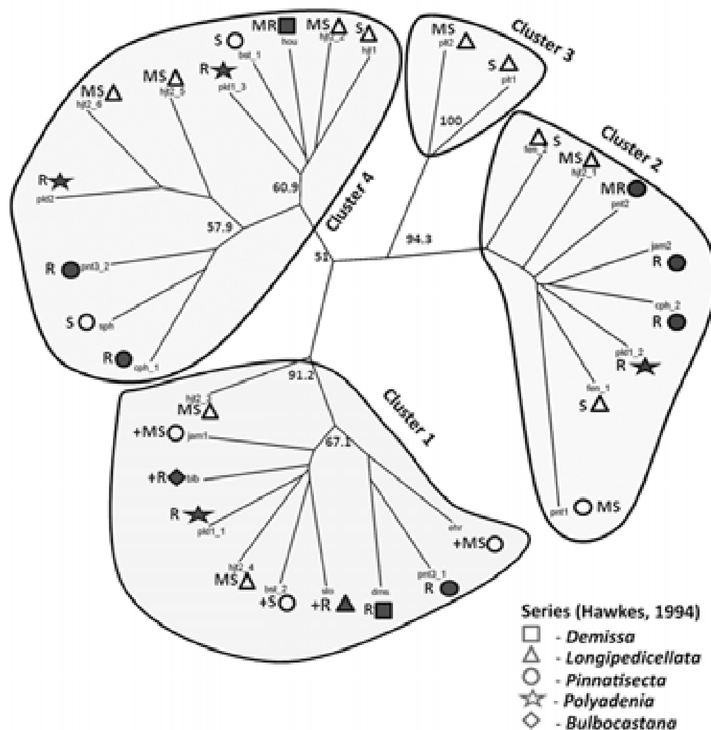

Figure 2. *Phylogenetic analysis (Maximum likelihood) of the RB fragments (RB-629).*
'+' – presence of allele-specific RB-226. LB resistance ranks are as follows: S – susceptible, MS – moderately susceptible, MR - moderately resistant, R – resistant. Filled are the pictograms of the resistant accessions. Bootstrapping was performed with 1000 replicates, and values higher than 50% are shown at the nodes. Cluster 1, haplotypes that joined with functional RB/Rpi-blb gene; cluster 2, pseudogenes, except for pnt2 haplotype; cluster 3, S. polytrichon-specific haplotypes; cluster 4, other haplotypes. For the list of sequences refer to Table 1.

CONCLUSIONS

Environmentally-friendly strategies for managing durable LB resistance are partially based on introgressing the *R* genes from wild *Solanum* species into commercial potato cultivars. Gene-specific markers proved to be effective and efficient tools when searching for orthologous *R* genes of broad-spectrum LB resistance, such as *RB/Rpi-blb1*, in wild *Solanum* species. Allele-mining approach helps to screen extensive collections of wild *Solanum* germplasm and to identify prospective candidates for comprehensive cloning experiments and potato introgression breeding for durable LB resistance. Using gene-specific SCAR markers, we found that *RB*-like structural homologues were universally distributed across wild *Solanum* species section *Petota*. An 18-bp long indel characteristic of functional *S. bulbocastanum RB* allele tagged by marker RB-226 was found in several *Solanum* species, and its presence was not always associated with high LB resistance. Sequencing and comparative analysis of *RB*-like gene fragments revealed ancient origins of the duplicated *RB*-like loci and characteristic patterns of nucleotide substitutions and indels most of which apparently arose before *Solanum* speciation.

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