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Concomitant reiterative BAC walking and fine genetic mapping enable physical map development for the broad-spectrum late blight resistance region, *RB*

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Abstract The wild potato species *Solanum bulbocastanum* is a source of genes for potent late blight resistance. We previously mapped resistance to a single region of the *S. bulbocastanum* chromosome 8 and named the region *RB* (for "*R*esistance from *S. Bulbocastanum*"). We now report physical mapping and contig construction for the *RB* region via a novel reiterative method of BAC walking and concomitant fine genetic mapping. BAC walking was initiated using RFLP markers previously shown to be associated with late blight resistance. Subcontig extension was accomplished using new probes developed from BAC ends. Significantly, BAC end and partial BAC sequences were also used to develop PCR-based markers to enhance map resolution in the *RB* region. As they were developed from BAC clones of known position relative to *RB*, our PCR-based markers are known a priori to be physically closer to the resistance region. These markers allowed the efficient screening of large numbers of segregating progeny at the cotyledon stage, and permitted us to assign the resistance phenotype to a region of approximately 55 kb. Our markers also directed BAC walking efforts away from regions distantly related to *RB* in favor of the 55-kb region. Because the *S. bulbocastanum* genotype used in BAC library construction is heterozygous for *RB* (*RB*/*rb*), codominant PCR-based markers, originally developed for fine-scale mapping, were also used to determine homolog origins for individual BAC clones. Ultimately, BAC contigs were constructed for the *RB* region from both resistant (*RB*) and susceptible (*rb*) homologs.

Keywords *Solanum bulbocastanum* · General resistance · Reiterative mapping · Cleaved Amplified Polymorphic Sequences (CAPS)

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Introduction

Worldwide production of the cultivated potato (*Solanum tuberosum* L.) exceeds that of all other dicot food crops (Food and Agriculture Organization, http://apps.fao.org/). Potato is also host to more than sixty pathogens of economic significance (Stevenson et al. 2001). Among the most devastating diseases of potato is late blight, a foliar and tuber disease caused by the oomycete *Phytophthora infestans*. A major cause of the Irish Potato Famine, which resulted in the death of one million people in the mid-19th century, late blight infestation can cause complete crop loss. Despite decades of active efforts by plant breeders to control this disease, late blight still causes the loss of billions of revenue dollars for growers each year (Kamoun 2001). The exploitation of genetic resistance remains the most promising approach for the long-term control of late blight.

Less adapted cultivated potato germplasm and the approximately 225 wild *Solanum* species are potential sources of genes for durable disease resistance for long-term potato protection. Jansky (2000) has summarized reported wild and cultivated sources of resistance to nine important potato diseases, including late blight. Among wild potato species with late blight resistance is the hexaploid species *S. demissum*. Resistance from this species was first incorporated into potato via sexual crosses nearly 100 years ago (Salaman 1911; Umaerus and Umaerus 1994). A series of genes, collectively referred to as the "R" series, has been described from this species (Black and Gallegly 1957; Malcolmson and Black 1966; Umaerus and Umaerus 1994). These genes are characterized by pathogen race specificity and condition a hypersensitive reaction to infection. Although R gene-mediated resistance showed great promise initially, the late blight pathogen quickly adapted to circumvent the plant's defenses (Toxopeus 1956; Black and Gallegly 1957). Kuhl et al. (2001) have described and mapped *Rpi1*, a late blight resistance gene from *S. pinnatisectum*. *Rpi1* has never been deployed for potato protection, and the durability of *Rpi1* -mediated resistance remains unexplored. Characterization of the *P. infestans* isolate used in that study led the authors to conclude that *Rpi1* might correspond to the pathogen race specific gene *R9* from *S. demissum* (Kuhl et al. 2001).

Late blight resistance genes from other wild *Solanum* species may offer different modes of resistance and broader control of the late blight pathogen. Of particular promise is the wild Mexican diploid species *S. bulbocastanum*. This species is highly resistant to late blight, and the possibility of exploiting this resistance for potato protection has long been recognized (Reddick 1939; Neiderhauser and Mills 1953; Graham et al. 1959). However, direct sexual crosses between 4EBN cultivated potato and the 1EBN *S. bulbocastanum* (Hanneman and Bamberg 1986) have never been reported. We have previously generated somatic hybrids between cultivated potato and *S. bulbocastanum* (Helgeson et al. 1998). Somatic hybrids retained the late blight resistance of the wild species, even under intense disease pressure and without fungicide protection. Significantly, the interspecific somatic hybrids were fertile and could be backcrossed (BC) to cultivated potato. Late blight resistance segregated in BC1 populations. Full resistance could be recovered even in advanced backcross generations, indicating that the somatic hybridization method could be used to transfer resistance from wild donor species to cultivated potato, despite the difficulties encountered with sexual crosses (Helgeson et al. 1998). Field tests with somatic hybrid-derived materials in the Toluca Valley of Mexico, where late blight pressures are extreme, suggested that resistance derived from *S. bulbocastanum* is race non-specific (Helgeson et al. 1998), unlike that associated with the previously deployed and long-since defeated R gene series from *S. demissum*. The successful generation of segregating somatic hybrid-derived backcross populations also made mapping experiments possible. These revealed a single map location on *S. bulbocastanum* chromosome 8 that was associated with the late blight resistance phenotype (Naess et al. 2000). This region was dubbed *RB* (for *R*esistance from *Solanum Bulbocastanum*).

The cloning of disease resistance genes from a variety of plant species in recent years has contributed to our understanding of how these genes are structurally arranged, revealed common motifs that are widely conserved among even divergent plant taxa, and raised the possibility of transferring resistance between genotypes, populations, and species via transgenic technology (reviewed by Hulbert et al. 2001). To date, the only published account of the successful isolation of a late blight resistance gene is that of Ballvora et al. (2002), who cloned the *R1* gene from *S. demissum*. The gene is of historical and scientific interest but, as it was long ago defeated by pathogen populations (Black and Gallegly 1957), it has limited potential application for long-term potato protection. We believe that the map-based isolation of *RB* will enable detailed analysis of its phenotypic functions, interesting comparisons of pathogen race specific (*R1*) and race non-specific (*RB*) late blight resistance genes, and future deployment for potato protection. With the ultimate goal of isolating *RB*, we have employed a reiterative method of large-insert clone isolation and genetic fine mapping, resulting in the construction of a physical map and a BAC contig for the *RB* region. This paper describes the procedure we followed.

Materials and methods

Plant materials and phenotypic assays

The generation of somatic hybrids between a single heterozygous (*RB*/*rb*) late blight-resistant genotype of *S. bulbocastanum* PI 243510 and the late blight susceptible cultivated potato accession PI 203900 (Helgeson et al. 1998) and segregating BC progeny from this somatic hybrid (Helgeson et al. 1998; Naess et al. 2000) have been reported previously. All genotypes were asexually maintained as tubers and as in vitro plantlets. Additional BC progeny were generated by crossing late blight-resistant somatic hybrid-derived materials with the susceptible potato cultivars Katahdin and Atlantic, or with the susceptible potato breeding line A89804-7. BC progeny were a gift from Dr. Joe Pavek (USDA-ARS, Aberdeen, ID) or were generated by our program. Protocols for phenotypic analysis of late blight resistance utilizing field and greenhouse tests at the University of Wisconsin (Madison) and field tests at the International Cooperative Program for Potato Late Blight (PICTIPAPA) in Metepec (Mexico) have been reported previously (Helgeson et al. 1998). For mapping purposes, we assayed 536 BC3 genotypes for resistance to *P. infestans* pathotype US8 in greenhouses at Madison. Individual genotypes were replicated an average of 3.9 times (range: 1–14). Seventy-five and 199 of these BC3 lines were subsequently field tested at PICTIPAPA in 1997 and 1999, respectively. A total of 542 BC2, 1060 BC3, and 206 BC4 genotypes were screened using molecular markers.

BAC library construction

The construction of a high-quality BAC library for *S. bulbocastanum* has been reported previously (Song et al. 2000). The library, prepared by partial digestion of genomic DNA with *Hin*dIII, was

augmented by the generation of a second library of 68,352 clones constructed from a partial digest of genomic DNA with *Bam*HI. These two libraries have average insert sizes of 155 kb and 125 kb, respectively, and together provide 14 × genome coverage. A third BAC library was constructed by complete digestion of genomic DNA with *Bam*HI, yielding a total of 8,448 clones in the BAC vectors pBeloBACII (Shizuya et al. 1992; 2304 clones) and pCLD04541 (Bent et al. 1994; 6144 clones). All three BAC libraries were prepared from the same single *RB*/*rb S. bulbocastanum* PI 243510 genotype used in the initial somatic fusion using previously reported methodologies (Song et al. 2000), except where noted. The BAC library was arrayed onto nylon filters (Hybond N+; Amersham Biosciences, Piscataway, NJ) using manual or robotic (QBot; Genetix, New Milton, Hampshire, UK) methods.

BAC library screening and contig construction via BAC walking

BAC membranes were screened via Southern hybridization (Southern 1975). Probes for Southern hybridizations included the tomato-derived genomic clones TG261 and TG495, the tomato-derived cDNA clones CT64 and CT88, and the RAPD marker G02—all markers previously shown to be associated with *RB*-mediated late blight resistance (Naess et al. 2000).

Additional probes were developed from the end sequences of selected BAC clones associated with the region via PCR amplification of BAC template DNA. PCR primers were designed from BAC end sequences using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Integrated DNA Technologies (Coralville, IA). Reactions were carried out in a total volume of 50 μl containing 1× reaction buffer containing 1.5 mM MgCl2, supplied by the manufacturer (Applied Biosystems, Foster City, CA), each dNTP at 0.2 mM, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), 50 ng of template DNA (BAC clone or genomic *S. bulbocastanum* DNA), and 50 pmol of each PCR primer. PCRs were carried out in a thermocycler (Applied Biosystems) under the following conditions: 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 52°C, and 1 min at 72°C. Aliquots (5 μl) of each completed PCR were visually characterized for amplification efficiency and correct product size following electrophoresis through a 0.8% TBE agarose gel, ethidium bromide staining, and visualization under a UV source. Provided reaction efficiency and accuracy were acceptable, the product of each reaction was purified using a MicroSpin S-200 HR column (Amersham Biosciences) according to the manufacturer's instructions. Five microliters of purified PCR product or 100-ng samples of tomato- and RAPD-derived fragments were labeled with ^{32}P using the DECAprime II Random Priming DNA Labeling Kit (Ambion, Austin, TX). Unless previously known to be low-copy number in nature, each probe was initially tested on a single BAC membrane containing 1,536 clones. Probes confirmed to be of low copy number were subsequently used to screen the entire collection of BAC clones using formamide-based hybridization (Sambrook and Russell 2001). Probes were used singly or were multiplexed in pairs or triples. Table 1 lists all probes used to screen the BAC libraries and, where applicable, primer sequence information for the generation of the probe fragment and amplicon size.

Table 1 Probes used for BAC contig initiation and extension

Probe[a]	Subcontig region[b]	Probe origin	Primer sequences (amplicon size) or reference
G02	G02	RAPD	GGCACTGAGG[c]
5O15F	G02	BAC end	CCCTTCTTATTCTTTAAGCAAACTT; ATTTTTCCCCTCCCAGCTT (107 bp)
5015R	G02	BAC end	ATTTTGCTCGAATCGCTCAT; TCCCAATTGGATCACTTGTCT (245 bp)
12B7F	G02	BAC end	TGTTTGTGCATTGAAGATTGG; TGGTAAGAAGGGCATTCCATA (162 bp)
32A7a	G02	BAC end	GGAAATACTAGAGGGGAGGGAGT; TGAATAAGCAGTTCGGTTTGAA (493 bp)
32A7b	G02	BAC end	TCTCTTGGGATCACGATTCA; TTTAATTTCGGCGGATGAAC (367 bp)
51H24F	G02	BAC end	TTTGGAGGATAGCAATACTTGGA; AGCAACTGGTGAGAAAATGTCTT (189 bp)
219D10F	G02	BAC end	CGGTTCAGCTGACCTTTCAT; ACCTGCGAGTGGATCAAAAC (103 bp)
CT88	CT88/TG495	Tomato cDNA	Naess et al. (2000)
TG495	CT88/TG495	Tomato genomic DNA	Naess et al. (2000)
7O17R	CT88/TG495	BAC end	TCACAATGCTAATATGTGGTTTGA; AGTTGTTTGTGGCTGCCATT (297 bp)
12F6F	CT88/TG495	BAC end	AGGTGTCCAAGTGAAAAGTCG; ATCAAGCACCTCCCCAAAC (170 bp)
49N10F	CT88/TG495	BAC end	AACTAGCCCGCGATCAACTA; AAACCGACACAGATGCAACA (365 bp)
52M2F	CT88/TG495	BAC end	CCCTCTGTTCCGTGACAAAT; CACAGAAGGGGGTTGATCTC (359 bp)
52M2R	CT88/TG495	BAC end	TGAGTTCCACAGTCTGTACATAACAA; TTTCTTCCTCTCCCTTCTCCTT (350 bp)
64K8R	CT88/TG495	BAC end	AACAAGATGAGCCTGGTGTG; ATCACATCCCAGAGGCAAAA (349 bp)
80G6F	CT88/TG495	BAC end	ATCAATCCATCATGTGAGCA; TCAGAAAATAAGCACGTTGACA (122 bp)
80G6R	CT88/TG495	BAC end	CTTGAGAAGGCAACGACAGA; GAAGGCGGGTAAACAGACAG (231 bp)
117J16F	CT88/TG495	BAC end	CAATCGCTCCTTCCAACTTC; TGAGCAGCATTCGAAGAAAA (361 bp)
122E4R	CT88/TG495	BAC end	AGGAATCTCCTCAAGTTCTACACA; GATACGGGTGCCAGGATTC (103 bp)
157M5F	CT88/TG495	BAC end	TTCAACCAGCAAGTTCAAGC; TTATTGTCCATGTCGCTCCA (357 bp)
201A16F	CT88/TG495	BAC end	GTTCCCATGCCTAAACCAGA; ATCGCCCGCTCAACTTAATA (115 bp)
201A16R	CT88/TG495	BAC end	TGAGGTATTGCTGTGGGTTG; TGAATTCAGCCCAGAAGTGAA (103 bp)
CT64	CT64/TG261	Tomato cDNA	Naess et al. (2000)
TG261	CT64/TG261	Tomato genomic DNA	Naess et al. (2000)
61A13R	CT64/TG261	BAC end	ATTCAATCGCCTGTCCAAAC; CATTGCTCTCGTTGGATGAA (333 bp)
103H7F	CT64/TG261	BAC end	CCCTCGACATGAACCAGAAG; TGTCCATGTAGGCCAAGACC (352 bp)
120C9F	CT64/TG261	BAC end	ACAGGCCAGGGTTCAAAATA; GCAATGGACAGACTTGATGC (378 bp)

[a]The chromosomal location of each probe is indicated in Fig. 2
[b]Subcontig regions are as defined in Fig. 2
[c]Operon G-02, Westburg BV, Leusden, The Netherlands; Naess et al. (2000)

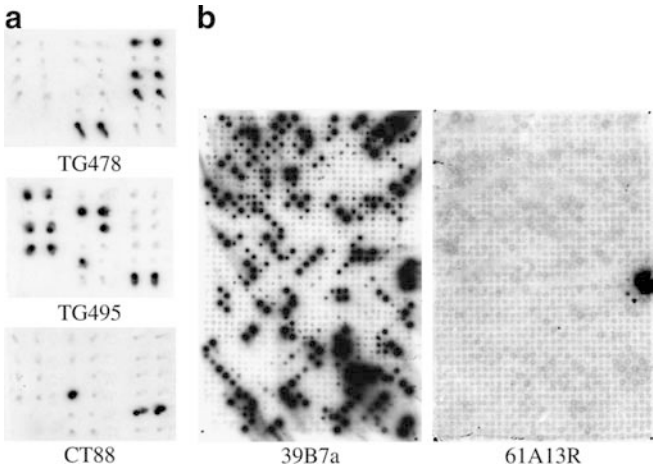

Fig. 1a, b BAC library screening. **a** For BAC library screening, probes were used singly or in multiplexed hybridizations of two or three probes. Single isolates of positive BAC clones were subsequently re-arrayed on membranes and re-hybridized to individual probes. Shown are three identical membranes prepared from putatively positive BAC clones initially identified in a multiplexed reaction with the RFLP probes TG478, TG495, and CT88. Re-arrayed membranes were screened with each probe individually, allowing conclusive assignment of some BAC clones to the RB region and to particular RFLP probes. **b** Probes developed from BAC ends for chromosome walking were first tested by hybridization to membranes containing DNA from 1,536 colonies. Approximately one in six BAC end probes associated with the *RB* region was multicopy (*left*) and could not be used for subcontig extension. Low-copy-number probes (*right*) were subsequently used to screen the BAC library en masse

The identity of single colony isolates for each positive BAC clone was confirmed by Southern hybridization using the same probe or probes used to detect them initially. If a group of clones had been identified in multiplexed Southern hybridizations, the confirmation step was repeated for each probe separately (Fig. 1). For positive BAC clones, an average of 552 bp of sequence information was generated from each end, using previously reported methods (Zhao et al. 2001).

BAC walking involved the reiterative screening of the BAC library using probes derived from the ends of previously identified BAC clones. Each probe identified multiple BAC clones. Clones were arranged into subcontig groups via cross hybridization of individual BAC ends; BAC ends present at the termini of the subcontig groups hybridized only to the BAC clones from which they were derived. PCR-generated probes from terminal BAC clones were subsequently used to screen the BAC libraries, as described above.

Insert size was estimated for selected BACs using the methods of Song et al. (2000). BAC clone 177O13 was partially sequenced using a shotgun approach as reported previously (Yuan et al. 2002).

Reiterative fine genetic mapping and determination of homolog origin

Cleaved Amplified Polymorphic Sequences (CAPS) and Sequence Characterized Amplified Region (SCAR) markers were developed from partial BAC sequences and BAC end sequences. PCR primers were selected manually or using Primer3 software to maximize amplicon size. PCR products were generated in a total volume of 25 μl containing 1× reaction buffer (Applied Biosystems), 2 mM MgCl2, 0.2 mM each dNTP, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), approximately 15 ng of genomic DNA, and 5 pmol of each PCR primer (Integrated DNA Technologies). Standard thermocycler (Applied Biosystems) conditions were 94°C for 2 min followed by 30 cycles of 94°C (1 min), 55°C (30 s), 72°C (90 s). When necessary, annealing temperatures were adjusted to match the predicted melting temperature of the primer pair.

Initially, primers were used to generate fragments from the *RB*/*rb S. bulbocastanum* PI 243510 genotype used in somatic hybridization and BAC library construction, the late blight-susceptible cultivated potato cultivar Katahdin or line R4, and a late blight-resistant and late blight-susceptible potato + *S. bulbocastanum* somatic hybrid-derived BC1 line. Polymorphic fragments were subsequently mapped to *S. bulbocastanum* chromosome 8 using a set of eight resistant and eight susceptible BC1 lines. For CAPS markers, entire PCRs were digested by the addition of 2 μl of an enzyme mixture composed of 2 U each of *Cfo* I, *Dra* I, *Rsa* I and *Taq* I (all from Promega, Madison, WI), or *Tfi* I (New England Biolabs, Beverly, MA) and 1× restriction enzyme buffer (supplied by the manufacturer), followed by incubation for 2 h at 37°C (*Cfo*I, *Dra* I, and *Rsa* I) or 65°C (*Taq*I and *Tfi* I). Digested products were separated by electrophoresis through a 0.6% agarose + 1.0% Synergel (DiversifieD Biotech, Boston, MA) gel in TBE buffer. Polymorphisms were visualized using ethidium bromide stain and UV irradiation. Polymorphic products with easily distinguished patterns were subsequently used to screen advanced BC populations.

DNA was extracted from single cotyledons of BC individuals using the microprep method of McGrath et al. (1994). All individuals were tested for the presence of markers flanking the resistance locus (G02 and 103H7R, or G02 and P09; Naess et al. 2000); recombinant individuals were tested with additional markers (CT88, 137E3R, 177O13R, 186A3F) to further pinpoint the recombination site. Table 2 summarizes CAPS and SCAR markers generated for fine mapping, including PCR primers, product size, and, where appropriate, polymorphic enzyme site. Map locations are indicated in Fig. 2. BAC clones associated with an approximately 55-kb region shown genetically to be responsible for the resistance phenotype were screened using genetically mapped CAPS or SCAR markers to determine homolog (*RB* vs. *rb*) origins (Fig. 2).

Results

Contig development and extension

Previous mapping of the *RB* phenotype revealed that a single region on *S. bulbocastanum* chromosome 8 was responsible for late blight resistance (Naess et al. 2000). This region explains 62.1% of the observed phenotypic variation, and no other genome location was significantly correlated with resistance. Initial linkage mapping of 114 BC2 individuals placed the resistance factor between RAPD G02 and RFLP CT64 (Naess et al. 2000; S. K. Naess, S. M. Wielgus and J. P. Helgeson, unpublished results). No recombination was observed between resistance and the RFLP markers CT88 and TG495 (Naess et al. 2000). In the present study, the markers G02, CT88, TG495, CT64, and TG261 were used as probes to initiate the construction of a physical map and a BAC contig encompassing the *RB* region.

Several BAC clones were identified with each probe in the libraries constructed from partial *Hin*dIII and *Bam*HI digests (Fig. 2). Following single-colony isolation and confirmation of identity, BAC clones were end sequenced and the sequence information was used to

Table 2 PCR-based markers developed for fine genetic Mapping of the *RB* region

Marker[a]	Marker type	Marker behavior[b]	Primer sequences	Fragment sizes (bp) [c]		
				Amplicon	*RB* homolog	*rb* homolog
52M2F	CAPS	Dominant	GAGGCAAACCCTCTGTTCCGT; GCTCCAAGTGGAGGAAATGCC	596	596	596
64K8R	CAPS	Dominant	AACAAGATGAGCCTGGTGTG; ATCACATCCCAGAGGCAAAA	349	349	349
103H7F	CAPS	Dominant	GCTTAGTGCCCTTAAGCG; CTGACTAACCGGATGGCC	549	299 + (37) + 213	299 + (37) + 213
137E3R	SCAR	Codominant	AAAATTGTCCTCCTCTAATTTTCTTT; TGATATGAAAAGAAAGTGGTTGC	750 [d] /307	750 [d]	307
162D4F	CAPS	Codominant	CGTGAAGTGAAATGCTCAACA; GCAAACTTTGGAAGGATTCG	569	569 [d]	(17) + 372 + 180
175F20F	SCAR	Dominant	CCTGAGCCTCGGTGAGAGTA; ACCCAAAACTCCCAACCTCT	353	353	353
177O13F	SCAR	Dominant	CTGGTTTGACAATGCTGGTG; GACACTCAAGGCTGCCATTT	598	598	598
177O13R	CAPS	Codominant	TCTGCAGAAAACCATCTCAGG; AGCTCTTAACACGCCTGGAA	400 [d] /380	400 [d]	380
186A13F	CAPS	Codominant	GCTTAAGCACGCTTCTGACA; TGACATGACCAGCCATTGAT	331	331	193 + 138
CAPS273C	CAPS	Dominant/ codominant[e]	CCCCAGAAAAGAACCCATCT; GCCGTCACCTCTGTCTTCTC	1008	*Taq*I: 1008/*Dra*I: 1008	*Taq*I: 1008/*Dra*I: 573 + 435
CAPS274A	CAPS	Codominant	AATTTCGGCCATTGAAAGAA; TTGGATGGCACTGATGTGAT	1012	1012	162 + 850
CT88	CAPS	Dominant	GTTGGGCAGAAGAGCTAG; TTGCCTTAGTCCCCAGAG	591	(70) + 136 + 385	(70) + 136 + 385
TG495	CAPS	Codominant	TGCAAGAGACACACATATGAC; AGCACTCTGTTCTCACAATTG	766/809 [f]	205 + 488 + (73)	244 + 488 + (77)

[a]The chromosomal location of each marker is indicated in Fig. 2

[b]Dominant markers distinguish between cultivated potato and *S. bulbocastanum* but do not distinguish between *RB* and *rb* homologs; codominant markers distinguish between *S. bulbocastanum RB* and *rb* homologs

[c]Fragment whose sizes are given in *parentheses* are not always visible on agarose gels

[d]Size is estimated from mobility on agarose gel

[e]*Taq*I reveals a dominant polymorphic site present in cultivated potato but lacking in the *S. bulbocastanum RB* and *rb* homologs; *Dra* I reveals a codominant polymorphic site present in the *S. bulbocastanum rb* homolog but absent in both cultivated potato and the *S. bulbocastanum RB* homolog

[f]The product of the *RB* homolog for TG495 CAPS is 766 bp in length; size polymorphism is readily distinguished in *Fok*I digests

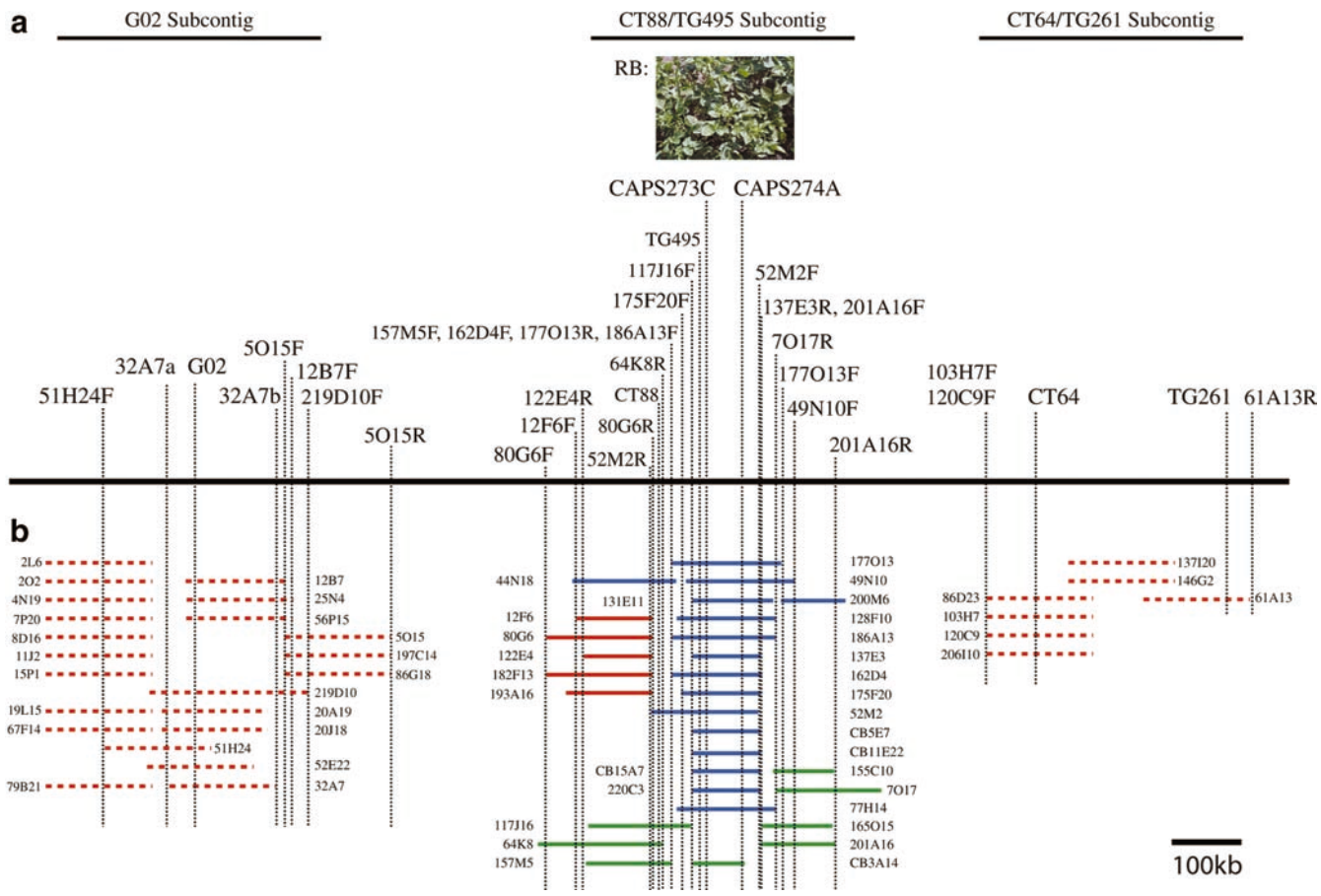

Fig. 2a, b Physical map of the late blight resistance region derived from *S. bulbocastanum*. **a** Integrated physical map of *S. bulbocastanum* chromosome 8 in the vicinity of *RB*. Genetically mapped markers include RFLP, RAPD, and BAC-end derived markers as indicated in Table 2. Marker locations are based on combined genetic and physical data for the region. All measurements between markers reflect actual or calculated physical distances. Subcontig groupings are indicated. Late blight resistance (*RB*) is mapped to a region flanked by the CAPS markers 273C and 274A, an area estimated to be 55 kb in length. **b** BAC contigs. BAC names indicate plate of origin and register location within a plate. BAC clones 1–64 originate from partial digestion of genomic DNA with *Hin*dIII; clones 65–240 originate from partial digestion of genomic DNA with *Bam*HI; clones with names preceded by "CB" originate from complete digestion of genomic DNA with *Bam*HI. Individual BAC clones are derived from the *RB* (resistant) homolog (*green*), the *rb* (susceptible) homolog (*blue*), or are of unknown homolog origin (*red*). BACs represented by *dashed lines* are of unknown insert size and their placement is tentative. Insert sizes have been estimated for BACs represented by *solid lines*; these BACs are drawn to scale (note 100-kb marker)

design new probes derived from each end of each BAC via PCR amplification. BAC end probes were subsequently hybridized to all overlapping clones (i.e. those BACs initially identified using a single, common probe) and the observed patterns of cross-hybridization were used to orient individual clones and order the entire subcontig grouping. Probes from the termini of the subcontig grouping, in theory, should hybridize only to the BAC clone from which they originated. With few exceptions, this method worked well for the *RB* region. In some cases, terminal probes contained sequence that was locally repetitive, and these probes could not be used to order subcontig groupings (not shown). Throughout the entire *RB* region, approximately one in every six BAC end probes was moderately to highly repetitive. Therefore, prior to screening the library en masse, we tested each probe using a single BAC filter of 1,536 clones (Fig. 1). Low copy number probes from the termini of BAC subcontig groupings (or internal to the grouping for cases in which terminal probes were repetitive) were used singly, in pairs, or in triples to screen the BAC library, extending subcontig clusters by BAC walking. Following isolation of individual colonies, all newly identified BAC clones were hybridized with each terminal BAC probe individually, both confirming clone association with *RB* and determining homology between each BAC and a specific probe.

Fine mapping using PCR-based markers of known physical proximity to *RB*

The generation of BAC end sequence data from *RB*-related BACs and a partial sequence from BAC clone 177O13 provided the opportunity to develop additional, PCR-based (CAPS and SCAR) markers for fine genetic mapping of the *RB* phenotype. The development and application of PCR-based markers greatly increased

marker density within the *RB* region (Fig. 2), allowing improved genetic resolution and substantially reducing the size of the interval for which contig development was necessary.

CAPS or SCAR markers more closely linked to resistance were developed from BAC sequences known a priori to be physically closer to the late blight resistance locus. That is, additional markers were developed from BAC sequences known to lie in the interval between the resistance locus and previously mapped markers. Using this reiterative process of concomitant subcontig extension and fine genetic mapping, it was possible to genetically restrict the factor(s) responsible for resistance to the region defined by RFLP TG495 and BAC end 52M2F (Fig. 2). BAC clone 177O13, which entirely encompasses the TG495/52M2F region, was subsequently selected for sequencing. CAPS 273C and CAPS 274A were ultimately developed from a partial sequence of 177O13. In a BC1 population of 118 individuals, one individual was recombinant between CAPS 273C and resistance, and another between CAPS 274A and resistance. This finding, and the absence of resistance gene candidates in the interval between CAPS 273C and RFLP TG495 and between CAPS 274A and BAC end 52M2F, led us to conclude that factors responsible for late blight resistance must reside in the interval between CAPS 273C and CAPS 274A. Based on subcontig models that incorporate calculated BAC sizes, we estimate the CAPS 273C/CAPS 274A interval to be approximately 55 kb in length. By "redefining" the *RB* region using this reiterative fine mapping approach, it was possible to focus physical mapping efforts exclusively on the CAPS 273C/CAPS 274A region. Thus, we suspended contig development of the G02 and CT64/TG495 subcontig groupings (Fig. 2).

The use of PCR-based markers allowed nondestructive assay of mapping individuals at the cotyledon stage. Individuals that were non-recombinant between markers flanking the resistance locus provided no added map resolution and were discarded, long before phenotypic analysis of late blight resistance was undertaken. This allowed rapid and cost-effective screening of greatly expanded mapping populations.

Homolog determination

BAC walking and reiterative fine mapping allowed efficient development of a BAC contig spanning the genetically defined *RB* region. However, because the single *S. bulbocastanum* genotype used in BAC library construction was heterozygous for late blight resistance (*RB*/*rb*), it was necessary to determine the homolog origin of all BACs in the immediate *RB* region, essentially separating BACs into an *RB* and an *rb* contig. Codominant CAPS and SCAR markers, previously used for fine genetic mapping, were invaluable for homolog determination. Assuming our BAC libraries represent an unbiased sampling of the *S. bulbocastanum* genome, 50% of BAC clones would be expected to originate from the *RB* homolog and 50% from the *rb* homolog. However, of 11 BAC clones encompassing the genetically defined 55-kb *RB* region isolated from the *Hin*dIII and *Bam*HI libraries, all, including the partially sequenced 177O13, were found to have originated from the susceptible *rb* homolog (Fig. 2). These BAC clones are not expected to carry alleles that impart late blight resistance. In contrast, BACs of known homolog origin flanking the genetically defined *RB* region showed segregation of homolog origin, with three of four and four of five BAC clones originating from the resistant *RB* homolog (Fig. 2). BAC libraries developed from partial digests of genomic DNA with *Hin*dIII and *Bam*HI were exhaustively screened for *RB* region clones.

Subsequently, a third BAC library was constructed from the same heterozygous *S. bulbocastanum* genotype. This library was prepared from a complete *Bam*HI digest of genomic DNA, reducing the sizes of fragments incorporating the genetically defined *RB* region to a minimum. Among the four BAC clones recovered from the newly prepared library using the RFLP probe TG495 was a single clone, of approximately 80 kb, which was derived from the resistant *RB* region. It is expected that this clone, BAC-CB3A14, carries alleles that impart late blight resistance in *S. bulbocastanum* and in the somatic hybrid-derived mapping populations.

Our results point to the possibility that the *RB* region is lacking in *Hin*dIII and *Bam*HI sites or is otherwise difficult to clone. It is anticipated that more detailed sequence analysis of the region may provide an explanation for our observations.

Discussion

Meksem et al. (1995) have stressed the importance of fine-scale genetic mapping as a prelude to map-based cloning efforts. In that study, the authors converted two RFLP markers that flank the late blight resistance *R1* locus into CAPS markers. The CAPS markers were then used to efficiently screen an expanded mapping population, identifying 17 recombinants in the region from among a total of 461 mapping individuals. An AFLP (Vos et al. 1995) bulked segregant analysis (Michelmore et al. 1991) ensued, identifying eight markers that were tightly linked to resistance (Meksem et al. 1995). The identification of these markers led to the successful isolation of the *R1* locus via BAC walking (Ballvora et al. 2002). Similarly, Zeng et al. (2002) employed RAPD bulked segregant analysis for fine genetic mapping and ultimate contig construction for the rice *Spl11* lesion mimic gene.

Here we report a reiterative method of concomitant fine-scale genetic mapping and BAC walking, and demonstrate its effectiveness by constructing a physical map and BAC contig for the *S. bulbocastanum* late blight resistance gene *RB*. Our work builds upon the CAPS method of Meksem et al. (1995). Rather than utilizing random marker systems, such as AFLP or

RAPD, to identify markers more closely linked to resistance, however, our reiterative method generates markers from BAC termini that are known to be physically closer to the trait of interest, and uses these to identify genetic markers that more precisely define the trait. Thus, our CAPS and SCAR markers are known a priori to be physically closer to the resistance locus than previously identified markers. Subsequent screening of the mapping population at large tests whether these markers are also genetically closer to the resistance determinant. The method proved particularly useful in our case, in which RFLP clones TG495 and CT88 had previously been shown to be associated, without recombination, with late blight resistance in a somatic hybrid-derived BC2 mapping population of limited size (Naess et al. 2000). Using TG495 and CT88 to screen our BAC library, we identified clones that were physically located within the G02-CT64 interval, allowing development of CAPS markers for fine genetic mapping in the immediate vicinity. Our reiterative method led to the ultimate genetic assignment of resistance to an interval of approximately 55 kb, and allowed definitive genetic placement of TG495 and CT88 relative to the resistance locus. Our method also allowed us to suspend expansion of the G02 and CT64/TG261 subcontig groupings in favor of the more precisely defined genetic region. It is expected that sequence analysis of the 55-kb region will readily identify the candidate resistance gene(s). In contrast, identification of candidate genes within the entire G02-CT64 interval, as would have been necessary in the absence of more detailed genetic mapping, would have been a daunting task. Thus, our reiterative method is useful not only for directing physical map development, but also for the ultimate identification of candidate genes. We agree with the conclusions of Meksem et al. (1995) that genetic fine mapping is important for map-based cloning efforts. Our research demonstrates that concomitant contig development and fine mapping is an efficient alternative or supplement to traditional fine mapping/map-based cloning approaches.

In our mapping population, polymorphisms between the resistant *RB* and susceptible *rb* homologs could be readily visualized as CAPS, or less frequently, as SCAR markers. Using a standard set of five frequently cutting restriction enzymes, four of seven CAPS markers developed from BAC end sequences for the late blight resistance region were found to distinguish individual *S. bulbocastanum* homologs; the remaining three CAPS markers distinguished *S. bulbocastanum* from cultivated potato, but could not differentiate individual homologs (Table 2). In contrast, of three SCAR markers developed for this region, only one distinguished between *S. bulbocastanum* homologs (Table 2). These observations support the possibility that point mutations, detected as CAPS polymorphisms, are more common than insertion/deletion events, detected as differences in amplicon size (i.e. SCARs). *Solanum bulbocastanum* is an outcrossing species that shows strong self incompatibility (Graham et al. 1959), resulting in population heterogeneity and a predominantly heterozygous genome for individual genotypes. Our reiterative method of identifying genetic markers based on known relative physical proximities could be adapted for sequencing-based polymorphism detection. This would enable the use of high throughput methodologies, and might be a more efficient approach for species, mapping populations, or genome regions with comparatively low levels of heterozygosity.

More than 30 disease resistance genes have been cloned from various plant species (Hulbert et al. 2001). The vast majority of these genes share a leucine rich repeat (LRR) with or without a nucleotide binding site (NBS) motif. Among known NBS-LRR disease resistance genes is the late blight resistance gene *R1* from *S. demissum* (Ballvora et al. 2002). The conservation of the NBS-LRR motif led to the development of a degenerate PCR method for generation of sequence from putative disease resistance genes (Leister et al. 1996). Originally developed for cultivated potato, the method has found widespread use in diverse plant species including both dicots and monocots (Collins et al. 1998, 2001; Pflieger et al. 1999; Fourmann et al. 2001; Penuela et al. 2002). Pan et al. (2000) generated and mapped more than 70 tomato sequences using this method. Interestingly, among the mapped NBS-LRR sequences are four fragments which map to the tomato chromosome 8 in close proximity to RFLP markers CT88 and TG495, a region corresponding to the *S. bulbocastanum RB* region. Given the documented synteny between tomato and potato (Bonierbale et al. 1988) and between potato and *S. bulbocastanum* (Brown et al. 1996) it will be interesting to determine whether *RB* is in fact homologous to NBS-LRR sequences generated by Pan et al. (2000).

Umaerus and Umaerus (1994) have summarized current opinion on the genetic nature of late blight resistance. Two broadly defined types of resistance have been recognized, race-specific resistance and general or field resistance. Race-specific resistance is exemplified by the *S. demissum R* genes. In addition to showing pathogen race specificity, this type of resistance is hypothesized to be conditioned by single genes that induce a hypersensitive reaction upon infection. When deployed on a large scale, race-specific resistance is thought to be short-lived and therefore of limited agricultural use, as was evident for the *S. demissum R1* gene. In contrast, general or field resistance is thought to lack a discernible hypersensitive phenotype and to be polygenic in nature, race non-specific, and durable. Late blight resistance from *RB* fits criteria of both types of resistance. Although race non-specific and lacking a discernible hypersensitive phenotype (Helgeson et al. 1998), *RB* maps to a single chromosome location of approximately 55 kb and may represent a single gene. Time alone can tell whether resistance conferred by *RB* will be durable. Our continuing efforts to delineate the *RB* locus offer the prospect for wide-scale deployment with potentially significant long-term benefits for potato production and the environment.

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