BAC end sequences corresponding to the B4 resistance gene cluster in common bean: a resource for markers and synteny analyses

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Abstract In common bean, a complex disease resistance (R) gene cluster, harboring many specific R genes against various pathogens, is located at the end of the linkage group B4. A BAC library of the Meso-american bean genotype BAT93 was screened with PRLJ1, a probe previously shown to be specific to the B4 R gene cluster, leading to the identification of 73 positive BAC clones. BAC-end sequencing (BES) of the 73 positive BACs generated 75 kb of sequence. These BACs were organized into 6 contigs, all mapped at the B4 R gene cluster. To evaluate the potential of BES for marker development, BES-derived specific primers were used to check for linkage with two allelic anthracnose R specificities Co-3 and Co-3², through the analysis of pairs of Near Isogenic Lines (NILs). Out of 32 primer pairs tested, two revealed polymorphisms between the NILs, confirming the suspected location of Co-3 and Co-3² at the B4 cluster. In order to identify the orthologous region of the B4 R gene cluster in the two model legume genomes, bean BESs were used as queries in TBLASTX searches of Medicago truncatula and Lotus japonicus BAC clones. Putative orthologous regions were identified on chromosome Mt6 and Lj2, in agreement with the colinearity observed between Mt and Lj for these regions.

Keywords Disease resistance gene · Phaseolus vulgaris · BAC-end sequence · Near isogenic lines · Synteny

Introduction

The legume family (Fabaceae) is one of the most agronomically and economically important plant families (Graham and Vance 2003). Of the legumes consumed directly by humans, half are common beans (Phaseolus vulgaris L.) (Broughton et al. 2003). Beans provide an important source of protein, vitamins (folate), and minerals (Ca, Cu, Fe, Mg, Mn, Zn) in human diets (Miklas et al. 2006). Diseases that reduce yields and seed quality are a major constraint on bean production, especially in developing countries where bean production often occurs under low input agriculture. Contributing factors include poor disease management, lack of resistant cultivars, and the limited availability of certified disease-free seeds. Consequently, development of resistant varieties is a primary goal of bean breeding programs throughout the world (Miklas et al. 2006). The use of resistant genotypes is not only an environmentally safe management strategy, but also a cost-effective strategy, especially for resource-poor farmers who cannot afford the purchase of pathogen-free seeds or pesticides.

Anthracnose, caused by the specialized hemibiotrophic fungus Colletotrichum lindemuthianum, is one of the most important diseases of common beans throughout the world (Pastor-Corrales and Tu 1989). The genetic basis of anthracnose resistance has been under study for nearly a century (Barrus 1911; Barrus 1915). The inheritance of
resistance follows the classic “gene-for-gene” model described by Flor (1955). Over 10 specific resistance (R) genes (Co- genes) conditioning resistance to different strains of *Colletotrichum lindemuthianum* have been characterized in common bean (for review see Miklas et al. 2006). Several of these have been mapped to the same region of the bean genome (Geoffroy et al. 2008). For example, the Meso-American *Co-9*, and the Andean *Co-z* and *Co-y* R specificities, present in BAT93 and Jal-oEEP558, respectively, are located at the end of the linkage group (LG) B4 (Geffroy et al. 1999). More recently, the *Co-3* gene, genetically characterized 30 years ago in the Meso-American bean genotype “Mexico222” (Bannerot 1965; Fouilloux 1976; Fouilloux 1979), has been localized to the same B4 region (Mendez-Vigo et al. 2005). Several years ago, a second allele, named *Co-3*² was also identified at the *Co-3* locus, in genotype “Mexico227” (Bannerot 1965; Fouilloux 1967, 1979). Consequently, at least five different anthracnose R specificities issued from different bean genotypes are clustered at the end of the LG B4. Furthermore, R specificities against diverse pathogens such as *Uromyces appendiculatus* (causative agent of bean rust), as well as against the bacteria *Pseudomonas syringae* pv. *phaseolicola* (causative agent of halo blight) are also present at the end of LG B4. Resistance QTL against bean golden yellow mosaic virus (BGYMV) and *C. lindemuthianum* have also been mapped to this area (Geoffroy et al. 2000; Lopez et al. 2003). Thus, the end of LG B4 carries a large set of specific R genes and R QTL effective against diverse pathogens. This pattern of R gene clustering is common in plant genomes (Pryor and Ellis 1993; Hulbert et al. 2001; Seah et al. 2007; Ashfield et al. 1998).

At the molecular level, most R genes encode proteins that contain both a nucleotide-binding site (NBS) domain and a leucine-rich repeat (LRR) domain (Meyers et al. 2005). NBS–LRR-mediated resistance has been identified against numerous types of biotrophic or hemibiotrophic pathogens, including fungi, bacteria, oomycetes and viruses, and these types of R proteins have been identified across a wide range of plant taxa (Jones and Dangl 2006). In the *Arabidopsis thaliana* genome, NBS–LRR sequences are often localized in complex clusters, suggesting that they derive from tandem duplications of an ancestral gene (Hulbert et al. 2001; Meyers et al. 2003; Leister 2004). Similar clusters of NBS–LRR-encoding genes have been identified in a large range of plant species, for example, the tomato *J2* locus (seven members in 90 kb) (Simons et al. 1998), the maize *rp3* locus (five members in 140 kb) (Webb et al. 2002), and the lettuce *Dm3* locus (32 members within at least 3,000 kb) (Meyers et al. 1998a, b; Kuang et al. 2004). In the common bean genome, one such NBS-LRR rich region has been identified at the end of LG B4, spanning a 2.7 cM interval in the vicinity of the *Co-9/Co- y* anthracnose R specificities (Geoffroy et al. 1999; Ferrier Cana et al. 2003; Ferrier Cana et al. 2005). Consequently, the B4 R gene cluster seems to be one of the most complex R gene clusters described in *P. vulgaris*, at both a genetic and molecular level.

Marker assisted selection (MAS) breeding has received much attention as a viable method for crop improvement. In the case of disease resistance genes, molecular markers linked to genes of interest can be used to accumulate R genes in a single cultivar without laborious disease screening (Miklas et al. 2006). However, costs associated with MAS are an important issue since they can be high. The development of molecular markers linked to a target gene represents an important part of that cost. The method known as bulked segregant analysis (BSA) (Michelmore et al. 1991), combined with highly polymorphic markers such as amplified fragment-length polymorphisms (AFLPs), has been successfully used in many plant species to identify molecular markers linked to the target gene (Mienie et al. 2005). However, this strategy can be time consuming and is not always successful at identifying tightly linked markers. Advances in high-throughput DNA sequencing chemistries have enabled new approaches to identifying polymorphic sequences linked to traits of interest. For example, short sequences, such as expressed sequenced tags (ESTs) or BAC end sequences (BESs), can now be easily generated and then screened for highly variable sequences such as simple repeats. Extensive EST and BES resources have been developed for many crop species, including papaya (Lai et al. 2006), wheat (Paux et al. 2006), wine grapes (Lamoureux et al. 2006), white clover (Febrer et al. 2007), banana (Cheung and Town 2007) and soybean (Nelson and Shoemaker 2006). These short sequences have been shown to be a powerful tool for developing molecular markers (Lopez et al. 2005; Lamoureux et al. 2006).

In preparation for sequencing of the B4 R gene cluster in common bean, we generated 114 BESs from 73 BAT93 BAC clones selected after hybridization with PRLJ1, a NBS probe previously shown to be specific to the B4 R gene cluster (Geoffroy et al. 1999). The analysis of these BESs revealed that 32 BESs had homology to the repeat sequence database or to large plant multigene families (such as NBS–LRR encoding genes). The remaining 82 BESs constitute a valuable resource for breeders who would like to develop PCR-based markers linked to one of the numerous R genes located at the B4 R gene cluster in different bean genotypes (Miklas et al. 2006). We demonstrated the validity of this approach for two anthracnose R specificities not present in our reference genotype BAT93, *Co-3* and *Co-3*². Finally, a TBLASTX similarity search was performed between the 82 selected BESs against the sequence of the two model legume genomes.
Medicago truncatula and Lotus japonicus in order to identify the orthologous regions.

Materials and methods

Plant material

An F9 recombinant inbred line (RIL) population, derived from the cross between an Andean landrace JaloEEP558 and a Meso-American breeding line BAT93 was used to map molecular markers. These 77 RILs have been used to set up an integrated linkage map of common bean. My and Mex222 carry the same resistance specificity Co-3, previously referred to as "Mexique Ia" (Bannerot 1965; Fouilloux 1976, 1979). Mz carries a second allele of Co-3, referred to as the Co-3^2 (Fouilloux 1979). Co-3^2 was previously referred to as "Mexique Ib" (Bannerot 1965; Fouilloux 1976, 1979). Two pairs of near-isogenic lines (NILs) differing at the Co-3 and Co-3^2 locus were a gift of Hubert Bannerot (INRA, Versailles, France). Co-3^2LVMy, Co-3^2LVMz and La Victoire. Co-3^2LVMy is derived from the introgression, via backcrossing, of the single major dominant gene Co-3 from the donor My into the Andean cultivar La Victoire (LV), which carries no known anthracnose resistance genes. In the same way, Co-3^2LVMz is derived from the introgression of the resistance specificity Co-3^2 derived from the donor Mz into La Victoire. Co-3^2LVMy is a BC5-F6 line and Co-3^2LVMz is a BC7-F5 line.

BAT93 BAC library screening

The NBS-PRLJ1 sequence was used as a probe in the hybridization experiments to screen the BAT93 BAC library (Geffroy et al. 1999; Kami et al. 2006). An aliquot of 25–30 ng of the purified fragment was labeled with 32P using the Ready-to-Go labeling kit (Pharmacia, Uppsala, Sweden). Prehybridization and hybridization steps were performed at 65°C in a solution containing 5 x SSC, 5% SDS, and 5 x Denhardt’s in a hybridization oven (Appligene, Illkirch, France). Three 20 minutes washes were performed in 0.5 x SSC and 0.5% SDS at 65°C. X-ray films (X OMAT-AR Kodak films, Rochester, NY, USA) were then exposed to the filters at −80°C using an intensifying screen. Putative positive clones were confirmed by PCR using the PRLJ1 primers (Geffroy et al. 1999).

BAC-end sequencing

BAC DNA was isolated using the plasmid maxi kit (QIAGEN, Courtaboeuf, France) according to the manufacturer’s guidelines. End sequencing of positive BAC clones was performed using an automated 3730A DNA Analyzer (Applied Biosystems, Roissy, France) with a reaction mixture composed of 8 μL of ABI Big Dye terminator V3.1 (Applied Biosystems, Roissy, France), 1 μL of template DNA, 1 μL of triton X100 1%, 8 pmol of the primer pIndigo forward (5'-CTCTGTGCTGCAAGGC GATTAAGTGTG-3') or pIndigo reverse (5'-CTCGTAT GTGGTGTGGAATTGGACG-3').

BAC-end sequence analysis

Sequence trimming was conducted using Sequencher Version 4.7 (Gene Codes Corporation, USA). BESs shorter than 150 bp were not retained for further analysis. Detection of redundant sequences was performed using the overlap program of the Genetics Computer Group (Madison, WI, USA) package (Devereux et al. 1984) and confirmed with an alignment using clustalX (Thompson et al. 1994). When a BES was 100% identical in its complete sequence with another longer BES, they were considered as redundant and only the longest one was further analysed.

BESs were screened for simple repeat sequences using REPEATMASKER (A. Smit, R. Hubley and P. Green, unpublished data; http://www.repeatmasker.org) with default parameters. Similarity with The Institute for Genomic Research (TIGR) Arabidopsis thaliana and legume repeat databases was searched with both BLASTN and TBLASTX to increase sensitivity using a cut-off e-value 10^{-03}. TIGR Arabidopsis and legume repeat databases were downloaded on April 3rd 2007 from TIGR (http://www.tigr.org/tdb/e2k1/plant.repeats/).

A non redundant set of 114 sequences was compared with the non-redundant protein database (nr) using the BLASTX program (e-value 10^{-06}) (http://www.ncbi.nlm.nih.gov/blast/). BESs without homology or with an e-value higher than 10^{-06} were annotated as “no homology”. The Munich information center for protein sequence (MIPS) functional classification was used to organize the bean BES into functional category: BES sequences were subjected to a homology search using BLASTX (cut-off e-value 10^{-06}) versus Arabidopsis MIPS database (http://mips.gsf.de/proj/thal/db/). In doing so, the assumption was made that functionality is transferable based on sequence conservation, to which there are many exceptions.

For comparative analysis, similarity with Lotus japonicus and Medicago truncatula BAC clone databases were searched with TBLASTX (cut-off e-value 10^{-08}). BESs presenting homology with large plant multigene families such as NBS-LRR, glycosyltransferase, receptor like protein (RLP), ST kinase or F-box encoding genes, as well as with the repeat database were excluded from this analysis.
Sequence databases were downloaded on April 3rd 2007 from NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) for *L. japonicus* and *M. truncatula* BAC sequence sets.

DNA extraction, primer design and PCR amplification

DNA extraction, PCR amplification and primer design were performed as described in Ferrier Cana et al. (2005). PCR primer design was optimized with the “amplify” software (http://engels.genetics.wisc.edu/amplify/). 254-G15F1 (5′-GTTGGTTTGTAGCATGTC-3′) and 254-G15F2 (5′-GTTGCAGGAGGATCTCC-3′) are two primers designed from the forward BES of BAT93 BAC clone “254-G15”. In the same way, GA-G16R1 (5′-AGCTACCTTTTCCACTAC-3′) and GA-G16R2 (5′-CAACAGCCACTCTAAGGGT-3′) are two primers designed from the reverse BES of BAT93 BAC clone GA-G16.

BAC fingerprinting and contig development

DNA fragments of positive BACs were measured manually in 1% agarose gel after total digestion of one microgram of BAC DNA with HindIII and EcoRI. Fragments observed on the agarose gel were compared between BACs to identify duplicated bands as potentially overlapping fragments. Southern analysis and hybridization experiments using PRLJ1 as a probe were performed as described in Geoffroy et al. (1999). PCR-based marker analysis was also used for BAC contig establishment. In order to be specific, the primer pairs used in these PCR experiments were derived from the 82 BESs left after elimination of the ones with homology to the repeat database or to large plant multigene families. Each primer pair was used in PCR experiments on the 73 positive BAC clones. The sequences of the 82 primer pairs are available upon request.

Linkage analysis

A PCR-based approach was used to map the BAC clones on the common bean integrated genetic map (Freyre et al. 1998). The BAC-end derived specific oligonucleotide primers were used to amplify genomic DNA of the 77 BAT93 × JaloEEP558 F9 RILs. Size and presence/absence polymorphisms were scored. The MAPMAKER software version 3.0 was used (Lander et al. 1987). Linkage groups were established with a LOD threshold of 3.0 and a recombination fraction of 0.3. Marker order was estimated with a LOD threshold of 2.0 based on multipoint “Compare”, “Order” and “Ripple” analyses. Distances between markers were presented in Kosambi centimorgans (cM) (Kosambi 1944).

Results

Identification of 73 BAC clones corresponding to the bean B4 R gene cluster and development of 75 kb of BAC-end sequence

PRLJ1, a NBS probe encompassing the P-loop to MHD region, was previously shown to be specific to the B4 R gene cluster (Geoffroy et al. 1999; Ferrier Cana et al. 2003, 2005). PRLJ1 was used as a probe to screen a BAC library of the bean genotype BAT93 (Kami et al. 2006), leading to the identification of 73 positive BAC clones. These 73 BAC clones were sequenced from both ends. In total, 152 sequencing reactions were performed, providing 114 high-quality non-redundant reads (GenBank accession numbers: FI159954 to FI160067). The average edited read length of the 114 non-redundant BESs was 666 bp, which provided a total of 75,937 bp of *P. vulgaris* sequence.

Simple sequence repeats (SSRs) and repetitive elements

Two SSRs of at least 16 nucleotides in length were identified in the 114 BESs. Both of these were tetranucleotide tandem repeats: poly(TAAA) was identified in BES 59-I22R and poly(TTTA) was identified in BES 200-I19F.

Comparison of the 114 bean BESs, in which all simple sequence repeats had been masked, to the Arabidopsis and Legume repeat database revealed 7 BESs (6%) with homology to known plant repetitive elements (Table 1; Fig. 1). Five of them belong to class I retrotransposons: 4 retrotransposons containing long terminal repeat (LTR), and one long interspersed repeat (LINE). Among the 4 LTR retrotransposons, three were further classified as Ty1-copia and one as Ty3-gypsy. Two other BESs with homology to plant repetitive elements correspond to class II DNA transposons: one mutator-like and one CACTA-like. No class III, centromere or telomere related sequences or rDNAs were identified.

Coding regions

BLASTX was used to screen the non-redundant (nr) protein database (cut-off e-value 10−06). Sixty-six (58%) BESs did not have a significant match at this threshold and thus were annotated as “no homology”. Forty-eight (42%) BESs had a significant homology in the nr protein database (Table 1). The percentage of the 48 BESs found in the different categories according to the MIPS functional classification scheme is presented in Fig. 1. “Cell rescue, defense and virulence” is the most represented functional category (17 BESs), mainly through a large representation of NBS-LRR encoding BESs (15) (Table 1). Two BESs (EN-M10F and 288-I10F) showed homology to RLP,
<table>
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<th>Homologya</th>
<th>Accession number</th>
<th>Organism</th>
<th>E-valueb</th>
<th>Functionnal categoryc</th>
<th>Length (bp)</th>
<th>GPd</th>
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<td>48-B10R</td>
<td>Raffinose synthase</td>
<td>AAD02832.1</td>
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<td>Metabolism</td>
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<td>Protein farnesyltransferase subunit beta</td>
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<td>Organism</td>
<td>E-value</td>
<td>Functionnal category</td>
<td>Length (bp)</td>
<td>GP</td>
</tr>
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</tr>
<tr>
<td>97-H20F</td>
<td>HMG-I and HMG-Y, DNA binding; Plant MuDR transposase; Zinc finger</td>
<td>ABE79123.1</td>
<td><em>Medicago truncatula</em></td>
<td>1,00E-13</td>
<td>Transposable elements</td>
<td>626</td>
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<tr>
<td>276-A23F</td>
<td>Integrase, catalytic region; Zinc finger, CCHC-type; Peptidase</td>
<td>ABE80010.1</td>
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<td>15-I19F</td>
<td>Integrase, catalytic region</td>
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<td>1,00E-10</td>
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</tr>
<tr>
<td>FY-H19F</td>
<td>Protein F12K21.14 (similar to copia-type polyprotein)</td>
<td>ABE77869.1</td>
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<td>1,00E-38</td>
<td>Transposable elements</td>
<td>450</td>
<td>ND</td>
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<tr>
<td>GL-F18R</td>
<td>Ribonuclease H; Glutathione S-transferase, C-terminal-like</td>
<td>ABE91721.1</td>
<td><em>Medicago truncatula</em></td>
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<td>Transposable elements</td>
<td>613</td>
<td>4</td>
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<tr>
<td>13-P17R</td>
<td>ATPase, E1-E2 type; Peptidase M, neutral zinc metallopeptidases</td>
<td>ABE8434.1</td>
<td><em>Medicago truncatula</em></td>
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<td>1-M8R</td>
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<td>48-B10F</td>
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<tr>
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<td>ABE94392.1</td>
<td><em>Medicago truncatula</em></td>
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<td>RST1</td>
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<td><em>Arabidopsis thaliana</em></td>
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<td>AAP74222.1</td>
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<td>E1-M23R</td>
<td>TIN15.2 (argonaute like subfamily)</td>
<td>AAF79718.1</td>
<td><em>Arabidopsis thaliana</em></td>
<td>5,00E-14</td>
<td>Unclassified proteins</td>
<td>797</td>
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</table>

*a* Blastx data  
*b* Cutoff E-value at 1E-6  
*c* MIPS data  
*d* Genetic Position, defined on Fig. 2  
*e* Not Defined, correspond to three “isolated” BAC clones  
*f* Correspond to BAC clones belonging to contig “C” spanning GP 2 and GP 3
which are transmembrane proteins with extracellular LRRs and a short cytoplasmic tail (Hammond-Kosack and Parker 2003). One of these two BES (EN-M10F) is most similar to an RLP subclass that contains known RLPs involved in PAMP perception (Ron and Avni 2004). The other one, BES 288-I10F is most similar to LeEIX2 (elicitor ethylene-inducing xylanase) from tomato, which is the first example of a RLP involved in PAMP perception (Ron and Avni 2004).

Assembly of BAC contigs

In order to organize the 73 positive BAC clones into contigs, standard agarose-based BAC fingerprinting, Southern blot hybridization using PRLJ1 as a probe and PCR-based marker analyses were used. The primer pairs used in these PCR experiments were derived from the 82 BESs left after elimination of the ones with homology to the repeat database or to large plant multigene families. This combination of approaches led to the identification of 6 BAC contigs (referred to as A to F; Fig. 2a) consisting of 13, 8, 18, 10, 13 and 6 BACs and covering 170, 225, 410, 255, 420 and 215 kb, respectively. PCR-based mapping experiments confirmed that these 6 BAC contigs mapped to the end of LG B4 at four different genetic positions (GP) referred to as GP 1, GP 2, GP 3 and GP 4. GP 1, GP 2 and GP 3 span 2.7 cM at the end of LG B4, while GP 3 and GP 4 are separated by 26.4 cM (Fig. 2a). Five BAC clones could not be placed in contigs, but two of these were mapped to GP 1 (BAC GA-G16) and GP 3 (EW-F5) (Fig. 2a). The three other BACs (237-G15, 241-G19 and FY-H19) could not be mapped because we were unable to find a polymorphism between BAT93 and JaloEEP558 in PCR-based markers derived from their respective BESs. Southern hybridization with PRLJ1 to all 73 BAC clones revealed a heterogeneous distribution of PRRLJ1-related sequences, with several BAC clones presenting a high number of bands compared to others (Fig. 2b, c).

Comparative mapping

To determine whether the bean B4 R gene cluster is conserved in other legume species, we used the low copy BESs, i.e., BESs left after elimination of the ones with homology to the repeat database or to large plant multigene families (such as NBS–LRR, Glucosyltransferase, RLP, ST kinase or F-box encoding genes), to search for shared synteny in the genomes of two model legumes Medicago truncatula and Lotus japonicus (Young et al. 2005). Eighty-two BESs, corresponding to about 50 kb of sequence, were compared with M. truncatula and L. japonicus BAC sequences by translated BLAST (TBLASTX). Sixteen BESs had significant similarities.
(cut-off e-value $10^{-08}$) with *M. truncatula* BAC sequences (Fig. 3a): nine on chromosome Mt6, two on chromosome Mt2, two on chromosome Mt5, and one on each of chromosomes Mt1, Mt3, Mt8. On chromosome Mt6, the 9 hits corresponded to three closely linked BAC clones at one end of chromosome Mt6: AC124951 (4), AC174342 (4) and AC159162 (1). More precisely, these 3 *M. truncatula* BAC clones are all located in the distal region of the short arm of chromosome Mt6 between the telomere and marker “002A02” (located at position “0 cM” on the genetic map of Mt6) (http://www.medicago.org/genome/map_chr.php?chr=6). When *L. japonicus* BAC sequences were considered, 20 bean BESs were found to have significant similarities (Fig. 3b): 18 on chromosome Lj2, one on chromosome Lj1 and one on a BAC clone whose location in Lj genome is not known. On chromosome Lj2 the 18 hits corresponded to 8 BAC clones: LjT13J14 (6), LjT30P12 (4), LjT13C22 (2), LjT47H13 (2), LjT23F22 (1),
The number of hits for each identified BAC clone is indicated in brackets. The genomic location of each BAC clone is indicated in square brackets. *Lj* BAC clones belonging to contig CM0201 are marked. **ND** Not defined. **R** resistance, **Co**-**3** resistance specificity, derived from **My** genotype; **Co**-**3** resistance specificity, derived from **Mz** genotype; **Co**-**3**. **R** resistance specificity derived from **My** genotype; **Co**-**3** resistance specificity derived from **Mz** genotype.

**A**

AC158842 (1) [44.9 cM]
AC159962 (1) [ND]
AC124951 (4) [0 cM]
AC136450 (1) [36.2 cM]
AC147001 (1) [57.3 cM]
AC147877 (1) [61.5 cM]
CT954231 (1) [20.2 cM]
CT027660 (1) [31.04 cM]
AC174542 (4) [-3 cM]

**B**

LjT37H21 (1) [67.3 cM] (contig CM0201)
LjT36H17 (1) [0 cM] (contig CM0201)
LjT23F21 (1) [50.1 cM] (contig CM0201)
LjT36E22 (1) [30.2 cM] (contig CM0201)
LjT37H21 (1) [9.6 cM] (contig CM0201)
LjT31H17 (1) [21.7 cM] (contig CM0201)
LjT16L07 (1) [19.3 cM] (contig CM0201)
LjT13J14 (6) [20.5 cM] (contig CM0201)
LjT13C22 (2) [21.7 cM] (contig CM0201)
LjT47H13 (2) [20.5 cM] (contig CM0201)
LjT30P12 (4) [19.3 cM] (contig CM0201)

**Fig. 3** Results of TBLASTX similarity searches between the 82 selected BESs and *Medicago truncatula* (a) and *Lotus japonicus* (b) genomes. The number of hits for each identified BAC clone is indicated in brackets. The genomic location of each BAC clone is indicated in square brackets. *Lj* BAC clones belonging to contig CM0201 are marked. **ND** Not defined. **R** resistance, **Co**-**3** resistance specificity, derived from **My** genotype; **Co**-**3** resistance specificity, derived from **Mz** genotype; **Co**-**3**. **R** resistance specificity derived from **My** genotype; **Co**-**3** resistance specificity derived from **Mz** genotype; **Co**-**3**.

**Lj** T36E22 (1), LjT37H21 (1), LjT16L07 (1). Three of these BAC clones (LjT13J14, LjT13C22 and LjT47H13) belong to a single BAC contig CM0201 (360 kb) located between 20.5 cM (via marker TM0201) and 21.7 cM (via marker TM0400) on Lj2 (Sato et al. 2008). Furthermore, a fourth BAC clone (LjT30P12) with 4 BESs hits is close to contig CM0201, since it is mapped at only 0.8 cM from CM0201. The other positive Lj2 BAC clones are spread on Lj2: LjT16L07 (mapped at 9.6 cM via marker TM0254), LjT36E22 (mapped at 30.2 cM via marker TM0338), LjT23F22 (mapped at 50.1 cM via marker TM0803), LjT37H21 (mapped at 67.3 cM via marker TM0132) (Fig. 3b). In conclusion, the regions with the highest level of shared synteny with the bean B4 gene cluster were found at the distal end of the short arm of chromosome M6 of *M. truncatula* and in an internal region of 360 kb on chromosome Lj2 in *L. japonicus*.

Using BESs to develop molecular markers linked to R genes belonging to the B4 cluster

We made the working hypothesis that BESs developed in the present study for the reference genotype BAT93 constitute the raw material to develop molecular markers linked to R genes located at the B4 R gene cluster in different bean genotypes. We tested this hypothesis for two anthracnose resistance specificities, **Co**-**3** (present in genotype **My**) and **Co**-**3** (present in genotype **Mz**), which have been described as two alleles of the same gene.
amplification products were observed between Co-3 and My and Mz while between LV and My (Fig. 4). For both primer pairs, amplification products were observed in My and Mz as well as between LV and My (Fig. 4). For both primer pairs, amplification products were observed between Co-3LVMy and My on one hand, and between Co-3LVMy and Mz on the other hand. Since NILs are, in theory, genetically identical except in the introgressed region, the latter data suggested that the observed amplification products in My and Mz are linked to Co-3 and Co-3\(^2\), respectively. For Co-3 R specificity, this result was confirmed through the analysis of another bean line also carrying Co-3, called Mex222. Indeed, Mex222 produced amplification products identical in size to Co-3LVMy and My with both primer pairs tested (Fig. 4a, b). Mapping experiments confirmed the location of these two BESs at the B4 R gene cluster: GA-G16R is co-located with Co-9 (mapped at GP 1) and 254-G15F mapped 2 cM from Co-9 at GP 2 (Geffroy et al. 1999) (Fig. 2a). Consequently, these experiments confirmed the inferred location of Co-3 at the B4 R gene cluster (Mendez-Vigo et al. 2005) and demonstrated that, as expected, the second allele of Co-3, named Co-3\(^2\) is also located at the B4 R gene cluster.

### Discussion

To better understand the molecular processes involved in the evolution of plant disease resistance gene clusters, we are preparing to sequence a complex disease resistance gene cluster located at the end of linkage group B4 in common bean. In the present work, we describe the screening of a BAC library (genotype BAT93) with PRLJ1, a NBS probe specific of the B4 R gene cluster. Organization of the 73 positive BAC clones into contigs led to the identification of six BAC contigs, all mapped to the B4 R gene cluster, confirming that PRLJ1-like sequences constitute a large multigene family specific to the end of linkage group B4. The PRLJ1-like sequences were heterogeneously distributed across the B4 R gene cluster, with several regions extremely rich in PRLJ1 family members separated by large intervening regions with few or no PRLJ1 sequences. Such an organization into “sub-clusters” of NBS-LRR sequences has also been observed in the potato R1 resistance gene cluster (Kuang et al. 2005) and the soybean Rpg1-b cluster (Ashfield et al. 2003).

One hundred and fourteen BESs corresponding to the bean B4 disease resistance gene cluster were generated, providing 75 kb of non-redundant sequences. BESs analysis revealed, as expected, that the B4 R gene cluster is composed of many NBS–LRR encoding genes but also of genes involved in other cellular function such as metabolism or cellular communication/signal transduction. Such colocalisation of R genes and genes with other function have been found for other R gene clusters such as the tomato Mi R gene locus (Seah et al. 2007). In order to be specific to the target region (B4 R gene cluster) for molecular marker development and comparative genomics analysis, one important aspect of our work, was to remove the BES with homology to the repeat database or to large plant multigene families (such as NBS–LRR, Glucosyltransferase, RLP, ST kinase or F-box encoding genes). Consequently, out of the 114 initially obtained BESs, 82, corresponding to approximately 50 kb of sequence, were selected for subsequent analysis.

In common bean, pyramiding of R genes has been recommended as an approach to provide long-term control of various diseases, including anthracnose (Young and Kelly 1997). Traditional breeding procedures are inefficient for pyramiding resistance genes due to the need for multiple inoculations. Consequently, molecular markers offer new opportunities for gene pyramiding in a single cultivar by MAS. However, the development of molecular markers linked to a gene of interest is sometimes difficult. For example, Correa et al. (2000) tested 605 random primers in a bulked segregant analysis (Michelmore et al. 1991) to identify only two molecular markers linked to a bean rust resistance block. In the present study, we demonstrated that BESs from BAC clones containing a complex cluster of R genes can be used for developing molecular markers linked to these R genes. Specifically, we developed markers for two anthracnose specificities, Co-3 [whose location within the B4 R gene cluster had been indirectly inferred (Mendez-Vigo et al. 2005)] and Co-3\(^2\), taking advantage of two pairs of NILs. Indeed, out of only 32 primer pairs tested, two primer pairs gave polymorphic PCR amplification products between the NILs (Co-3LVMy/LV and Co-3\(^2\)LVMy/LV). Consequently, the BESs developed in the present study constitute the raw material for breeders to develop additional molecular markers linked to numerous R genes that have been previously mapped to the B4 R gene cluster (Geffroy et al. 1999; Miklas et al. 2006). Such a focused approach has been successfully used for the development of markers linked the rust resistance gene Rph7 in barley (Mammadov et al. 2007), taking advantage of a 226 kb sequenced region developed in a previous study (Scherrer et al. 2005). In the case of the B4 R gene cluster, one limitation of this approach seems to be the low level of polymorphism for this part of the bean genome.
Indeed, it should be noted that, in our study, only two out of 32 primers tested revealed polymorphism between “La-Victoire” (Andean) and “My” (Meso-american) and “Mz” (Meso-american). This level of polymorphism is substantially lower than usually observed between Andean and Meso-American genotypes (Nodari et al. 1992).

Our results confirmed that the Co-3 anthracnose R specificity is located within the B4 R gene cluster. Its location had been indirectly inferred through an allelism test between genotype “Mexico222” (Co-3) and “PI207262” (Co-9), revealing that Co-3 specificity is located in the vicinity of the Co-9 R specificity (Mendez-Vigo et al. 2005). We also demonstrated that another “allele” of Co-3, identified by Fouilloux (1976) 30 years ago, and referred to as Co-3⁵, is, as expected, also located within the B4 R gene cluster. Consequently, the B4 R gene cluster contains at least 5 different anthracnose specificities (Co-9, Co-y, Co-z, Co-3, Co-3⁵) against the hemibiotrophic fungus C. lindemuthianum, as well as R specificities against bacteria and another fungus, Uromyces appendiculatus (Miklas et al. 2006). At the molecular level, a family of NBS-LRR sequences has been identified at the B4 R gene cluster (Ferrier Cana et al. 2003; Ferrier Cana et al. 2005). The present data confirmed that PRLJ1 is a large family specific to the end LG B4 with more than 35 members present in genotype BAT93 (Fig. 2a). In plants, intraspecific variation in the copy number of NBS-LRR genes within an R cluster is frequently observed, probably resulting from unequal crossing over between tandemly arrayed genes (Chin et al. 2001; Kuang et al. 2004). Thus, it is not possible to identify true “allelic” resistance specificities among members of a complex R gene cluster. Consequently, we believe it is more appropriate to say that Co-9, Co-y, Co-3, Co-3⁵ are all members of the B4 R gene cluster rather than speculate on allelism relationships.

The public availability of data generated from two legume genomics programs makes possible the comparative analyses of the bean B4 R gene cluster with the two model legume species: M. truncatula and L. japonicus (Young et al. 2005; Cannon et al. 2006; Sato et al. 2008). Conceptual translations of bean BES DNA sequences (TBLASTX) were used for the comparisons because they provide a more sensitive test of homology between evolutionary distant species than do nucleotide sequence comparisons (Grant et al. 2000). A putative orthologous region was identified at the end of the short arm of chromosome Mt6 (3 Mt BAC clones presenting 9 significant hits with bean BESs) and in chromosome Lj2 (one 360 kb contig “CM0201” and BAC LjT30P12 closely linked to CM0201, presenting a total of 14 significant hits with bean BESs). This is in agreement with previous macro- and microsynteny analysis between L. japonicus and M. truncatula where it was shown that the short arm of chromosome Mt6 corresponds to part of chromosome Lj2 (Choi et al. 2004; Zhu et al. 2005; Cannon et al. 2006). More precisely, Cannon et al. (2006) reported that only 2.8% of chromosome Mt6 was covered by chromosome Lj2. In the consensus map for eight legume species presented in Zhu et al. (2005) chromosome Mt6 is unusual because no macro-synteny was detected for much of its sequence with other legume species including Phaseolus vulgaris (Choi et al. 2004; Zhu et al. 2005). Consequently, the present work is a contribution to the improvement of this consensus map through the establishment of shared syntenic relationships between the end of bean LG B4 [corresponding to linkage group B on the map established by Vallejos et al. (1992)] and the short arm of Mt6 and part of Lj2. Other features of note regarding chromosome Mt6 are that it is substantially more heterochromatic than the rest of the M. truncatula chromosomes and that it is also home to novel repeats not found elsewhere in the M. truncatula genome (Zhu et al. 2002; Kulikova et al. 2004; Cannon et al. 2006). Interestingly, no NBS–LRR encoding genes have been identified at the end of the short arm of chromosome Mt6 (Ameline-Torregrosa et al. 2008), as well as in the 360 kb “CM0201” Lj2 contig, suggesting a rapid reorganization of NBS-LRR sequences during legume genome evolution, similar to what has been described for cereal genomes (Leister et al. 1998).

This paper constitutes a first glimpse towards the understanding of the evolution of thebean B4 R gene cluster. The 73 bean BAT93 BAC clones, positive with the NBS-PRLJ1 probe, have been organized into contigs and BAC clones defining the minimum tilling path will be sequenced. This will allow us to infer the level of micro-synteny between the bean B4 R gene cluster and the two model legume genomes M. truncatula and L. japonicus as well as with the recently available soybean whole genome shotgun sequence.

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