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ORIGINAL PAPER

Fine mapping of *Co-x*, an anthracnose resistance gene to a highly virulent strain of *Colletotrichum lindemuthianum* in common bean

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Abstract

Key message **The *Co-x* anthracnose R gene of common bean was fine-mapped into a 58 kb region at one end of chromosome 1, where no canonical NB-LRR-encoding genes are present in G19833 genome sequence.**

Abstract Anthracnose, caused by the phytopathogenic fungus *Colletotrichum lindemuthianum*, is one of the most damaging diseases of common bean, *Phaseolus vulgaris*. Various resistance (R) genes, named *Co-*, conferring race-specific resistance to different strains of *C. lindemuthianum* have been identified. The Andean cultivar JaloEEP558 was reported to carry *Co-x* on chromosome 1, conferring resistance to the highly virulent strain 100. To fine map *Co-x*, 181 recombinant inbred lines derived from the cross between JaloEEP558 and BAT93 were genotyped with polymerase chain reaction (PCR)-based markers developed using the genome sequence of the Andean genotype G19833. Analysis of RILs carrying key recombination events positioned *Co-x* at one end of chromosome 1 to a 58 kb region of the G19833 genome sequence. Annotation of this target region revealed eight genes: three phosphoinositide-specific phospholipases C (PI-PLC), one zinc finger protein and four kinases, suggesting that *Co-x* is not a classical nucleotide-binding leucine-rich encoding gene. In addition, we identified and characterized the seven members of common bean PI-PLC gene family distributed into two clusters located at the ends of chromosomes 1 and 8. *Co-x* is not a member of *Co-1* allelic series since these two genes are separated by at least 190 kb. Comparative analysis between soybean and common bean revealed that the *Co-x* syntenic region, located at one end of *Glycine max* chromosome 18, carries *Rhg1*, a major QTL contributing to soybean cyst nematode resistance. The PCR-based markers generated in this study should be useful in marker-assisted selection for pyramiding *Co-x* with other R genes.

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Introduction

Common bean (*Phaseolus vulgaris*) is one of the most important staple foods in many parts of the world, particularly in developing countries where it constitutes the main source of protein for human consumption (Broughton et al. 2003). Unfortunately, bean production can be drastically impaired by environmental conditions and particularly by fungal diseases. Anthracnose, caused by the hemibiotrophic fungal pathogen *Colletotrichum lindemuthianum*, is one of the most widespread and economically important diseases

of common bean, especially in tropical bean-producing regions of Latin America and Eastern Africa where climatic conditions favor disease development and can result in total yield loss (Pastor-Corrales and Tu 1989). Because chemical control is expensive and generation of pathogen-free seeds is often difficult in developing countries, use of resistant genotypes appears to be the most biologically safe and cost-effective management strategy (Pastor-Corrales and Tu 1989).

Plant resistance to microbial pathogens is a complex process relying on different levels of resistance (Dangl et al. 2013). The genetics of anthracnose resistance in common bean has been studied for a long time, since this host/pathogen interaction was the first report of race-cultivar specificity (Barrus 1911, 1915; McRostie 1919). This so-called specific resistance relies on the specific recognition of pathogen-derived effectors, called Avirulence (Avr) proteins, by plant resistance (R) proteins encoded by R genes. Strikingly, regardless of the plant or the pathogen considered and despite the diversity of pathogen Avr proteins, the majority of cloned R genes encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Meyers et al. 1999; Dangl and Jones 2001; McHale et al. 2006). These proteins contain a central nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain (Michelmore et al. 2013). NB-LRR proteins can be divided into two subclasses based on N-terminal domains (Ameline-Torregrosa et al. 2008; Bai et al. 2002; Meyers et al. 2003): the TIR-NB-LRR (TNL) containing a *T*oll *I*nterleukin-1 *R*eceptor domain (Hammond-Kosack and Jones 1997) and the CC-NB-LRR (CNL) with a *C*oiled-*C*oil domain (Pan et al. 2000).

In plant genomes, NB-LRR sequences are often organized in complex clusters comprised of many tightly linked genes (Andolfo et al. 2013; Jupe et al. 2012; McDowell and Simon 2008; Meyers et al. 2003). In common bean, a complex disease R gene cluster, the *B4* cluster, located at the end of the short arm of chromosome 4 has been identified. The *B4* cluster consists in tandem array of CNL sequences (David et al. 2009; Ferrier Cana et al. 2003, 2005; Geffroy et al. 2009) and was genetically shown to harbor many specific R genes against various pathogens. Particularly, five R specificities effective against *Colletotrichum lindemuthianum* (*Co-3*, *Co-9*, *Co-y*, *Co-z* and *Co-10*) have been identified at this complex *B4* resistance cluster (Bannerot 1965; Geffroy et al. 1999; Mendez-Vigo et al. 2005; David et al. 2008; Miklas et al. 2006). Another complex cluster containing both CNL and TNL sequences was identified at the end of chromosome 11 (Creusot et al. 1999; Geffroy et al. 1998; Chen et al. 2010; Innes et al. 2008) where the anthracnose R gene *Co-2* was previously mapped (Adam-blondon et al. 1994; Mastenbroek 1960). Anthracnose R gene *Co-u* was mapped at one end of linkage group (LG) B2 (Geffroy et al. 2008) in a third cluster comprised of R genes to viruses (Vallejos et al. 2006). TNL sequences were identified in the vicinity of this cluster (Vallejos et al. 2006).

Another complex R gene cluster was located on LG B1 (Miklas et al. 2006). This cluster contains six R specificities against *C. lindemuthianum*: *Co-1*, *Co-1*^{2}, *Co-1*^{3} (Melotto and Kelly 2000), *Co-1*^{4} (Goncalves-Vidigal et al. 2011), *Co-1*^{5} (Goncalves-Vidigal and Kelly 2006) described as an allelic series, and *Co-w* (Geffroy et al. 2008). Two other R specificities against bean rust, caused by *Uromyces appendiculatus*, *Ur-9* (Jung et al. 1998; Park et al. 1999) and angular leaf spot, caused by *Pseudocercospora griseola*, *Phg-1* (Goncalves-Vidigal et al. 2011) were also mapped in this region of LG B1. However, unlike the aforementioned *B4*, *Co-2* and *I* clusters, the molecular basis underlying this complex R cluster remains unknown.

In a previous study, the *Co-x* anthracnose R gene, which confers resistance against the strain 100 of *C. lindemuthianum* in the Andean genotype JaloEEP558, was mapped to this complex cluster at the end of LG B1 (Geffroy et al. 2008). *Co-x* is an agronomically important R gene as *Co-x* confers resistance to strain 100, which is virulent on G 2333, a germplasm accession of Mexican origin, resistant to a broad range of *C. lindemuthianum* strains (Pastor-Corrales et al. 1994). Recently, the common bean genome has been sequenced and annotated (available since July 2012 via www.phytozome.org; Schmutz et al. 2014). The selected common bean genotype is "G19833", an Andean landrace for which a BAC library was used to construct a draft physical map (Schlueter et al. 2008). Access to the complete genome sequence provides the opportunity to rapidly develop locus-specific markers and has therefore a revolutionary impact on fine mapping strategies.

In the present report, we used a population of recombinant inbred lines (RILs) derived from a cross between BAT93 × JaloEEP558 to initiate the positional cloning of *Co-x* R gene. PCR-based markers developed in this study using common bean genome sequence have been used for fine mapping analysis and enabled us to delimit the *Co-x* locus into a 58 kb genomic DNA region that contains eight candidate genes. In this study, we also characterized the small family of seven phosphoinositide-specific phospholipases C (PI-PLC) present in common bean genome, of which three members are potential *Co-x* candidate genes.

Materials and methods

Plant and fungal material

A population of 181 F11 RILs, derived from the cross between the Andean landrace JaloEEP558 and the

Mesoamerican breeding line BAT93, was used to map the *Co-x* anthracnose resistance gene. This population is composed of 77 RILs used to set up the integrated linkage map of common bean (Freyre et al. 1998) and 104 additional RILs developed in Orsay (Chen et al. 2010). "La Victoire" is a French multi-susceptible common bean cultivar of Andean origin developed by the seed company "Tezier" (Valence-sur-Rhone, France). G19833, a CIAT germplasm accession, is a landrace originally collected in Peru that belongs to the Andean gene pool of common bean. The *Colletotrichum lindemuthianum* strain 100 was isolated from cultivated common bean in Costa Rica (Geffroy et al. 2008). A set of 12 differential cultivars from CIAT was used for pathotype characterization of strain 100: MDRK (*Co-1*), Kaboon (*Co-1²*), Perry Marrow (*Co-1³*) (Melotto and Kelly 2000), Widusa (*Co-1⁵*) (Goncalves-Vidigal and Kelly 2006), Cornell49242 (*Co-2*) (Mastenbroek 1960), My (*Co-3*), Mz (*Co-3²*) (David et al. 2008), TO (*Co-4*) (Fouilloux 1979), G 2333 (*Co-4²*, *Co-5²*) (Vallejo and Kelly 2009), PI207262 (*Co-4³*) (Alzate-Marin et al. 2007), TU (*Co-5*) (Fouilloux 1979; Young and Kelly 1996; Campa et al. 2009) and AB 136 (*Co-6*, *co-8*) (Alzate Marin et al. 1997; Young and Kelly 1996) (Supplementary Table S1).

Growth conditions and *C. lindemuthianum* pathogenicity test

The *Co-x* anthracnose R gene from JaloEEP558 and conferring resistance to *C. lindemuthianum* strain 100 was previously mapped at the end of LG B1 using the first set of 77 RILs described above (Geffroy et al. 2008). In the present study, strain 100 disease scoring was done on the 104 additional RILs as described in Geffroy et al. (2008). In brief, *C. lindemuthianum* strain 100 was grown at 20 °C in darkness for ≈10 days in plates containing potato dextrose agar medium. The inoculum was prepared by harvesting conidia by flooding *C. lindemuthianum* plates with distilled sterile water. The spore concentration was measured using a hemocytometer and adjusted to 5.10^6 spores/ml. For each RIL, six seeds were planted in pots filled with moist vermiculite. The parental lines BAT93 and JaloEEP558 and the highly susceptible cultivar "La Victoire" were included as controls. Seedlings were grown at 23 °C, 75 % relative humidity, with 8 h dark and 16 h light photoperiods under fluorescent tubes (166 µE). Eight days after germination, seedlings were sprayed with the 5.10^6 spores/ml suspension of *C. lindemuthianum* strain 100 and incubated at 19 °C, 90 % controlled humidity, under the same light conditions as described for the germination of seedlings. Symptoms were scored 6 and 7 days after inoculation. Plants without visible symptoms or showing very limited necrotic lesions were scored as resistant. Plants with large, sporulating lesions and dead plants were scored as susceptible. Two replicates of this experiment were carried out, i.e., the resistance/susceptible phenotype was assessed on at least 12 seedlings for each RIL.

DNA extraction

Nuclear DNA was isolated as described in Geffroy et al. (1998). In brief, 2 g of leaves were ground in liquid nitrogen. The powdered tissue was transferred into 50 ml of cold buffer 1 (10 mM Tris pH 7.5; 15 mM KCl; 15 mM NaCl; 0.15 mM spermine; 0.5 mM spermidine; 2 mM EDTA; 0.5 % triton-X100 and 4.5 % glucose) and filtered through blutex. The filtrate was centrifuged for 10 min at 2,600 rpm and 4 °C and the supernatant was discarded. The pellet was rinsed with 5 ml of buffer-1 three times and was finally resuspended into 1 ml of distilled water. Afterwards, a CTAB extraction procedure was performed as described in Doyle and Doyle (1987) except that the chloroform–isoamyl alcohol extraction step was replaced by a chloroform–octanol (24:1) extraction step.

PCR-based marker design and RILs genotyping

PCR-based markers were designed using the "*Phaseolus vulgaris* v1.0" reference genome sequence of G19833 at the Phytozome database (http://www.phytozome.net/commonbean.php) using Primer3 version 4.0 software (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky 2000) with default parameters. We designed 55 primer pairs (listed in Supplementary Table S2) targeted to intergenic regions as those regions present higher frequencies of polymorphism than coding regions. Primer pairs were designed to amplify about 1 kb with a GC percent of ~50 % and were selected to be locus specific. Primer specificity in the *P. vulgaris* genome was checked using custom Perl scripts based on the SPADS program (Thareau et al. 2003). All the primers were synthesized by Eurogentec Compagny (Liege, Belgium). These primer pairs were experimentally tested for amplification and polymorphism between the parental lines BAT93 and JaloEEP558. PCR reactions were performed using Go Taq DNA Polymerase kit (Promega, Charbonnières-les-Bains, France) in a standard PCR program (start: 94 °C for 5 min; amplification: 35 cycles of 94 °C for 30 s, 59–65 °C (detailed in Supplementary Table S2) for 45 s, 72 °C for 1–2 min; termination for 5 min). PCR products were resolved on 1 % agarose gels containing ethidium bromide, run in 0.5× TBE buffer and visualized under UV light. Primer pairs revealing polymorphism between BAT93 and JaloEEP558 were used to genotype the 181 RILs following the same PCR protocol as described above. To identify coordinates of PCR-based markers CV542014 and TGA1 (Goncalves-Vidigal et al.

2011) forward and reverse primers sequences of CV542014 and TGA1 (Supplementary Table S2) were used as query in BLASTN analysis against G19833 genome (www.phytozome.org).

Genetic mapping

A Chi-squared (χ^2) test was used to evaluate the goodness of fit of observed and expected segregation ratio. The Mapmaker software version 3.0 (Lander et al. 1987) was used to map the *Co-x* resistance gene and the PCR-based markers on the integrated linkage map of common bean using the set of 142 makers (Geffroy et al. 2000) as described in Geffroy et al. (2008). Linkage groups were established with a LOD threshold of 3.0 and a maximum recombination fraction of 0.3. Markers order was estimated with a LOD threshold of 2.0 based on multipoint "compare", "order" and "ripple" analyses. Map distances were estimated by the Kosambi mapping function (Kosambi 1944).

Sequence annotation

The targeted region was annotated using an automatic annotation pipeline described in David et al. (2009). The pipeline uses a combination of gene-finding programs and sequence homology with known genes and proteins: the two ab initio gene prediction programs FGENESH (Burset and Guigo 1996) and GeneMarkhmm (Lukashin and Borodovsky 1998), BLAST (Altschul et al. 1997) analyses against the GenBank nonredundant database and all the *Phaseolus* ESTs available at GenBank (Ramirez et al. 2005). All this information was imported into the annotation platform Artemis (Rutherford et al. 2000) for further manual analysis and compared with the annotation provided in Phytozome.

Phylogenetic analysis of the PI-PLC gene family

To identify PI-PLC genes in the G19833 genome, we performed a BLASTP search (Altschul et al. 1997) using Phvul.001G243100, Phvul.001G243200 and Phvul.001G243300 predicted protein sequences as query against G19833 genome sequence (www.phytozome.org). For each PI-PLC coding gene identified, annotation and intron–exon structure were manually checked. Multiple amino acid sequence alignment of full-length PI-PLC protein sequences was generated using MUSCLE (Edgar 2004a, b) with default parameters and edited in GENEDOC for manual adjustments (Supplementary Figure S2). A maximum likelihood tree was made with MEGA version 5 (Tamura et al. 2011). Bootstrap values were computed with the consensus of 1,000 random trees. The resulting phylogenetic tree (Fig. 3c) was displayed with MEGA.

Common bean and soybean synteny analysis

Soybean (*Glycine max*) v1.1 (Schmutz et al. 2010) and common bean G19833 v1.0 (Schmutz et al. 2014) genome sequences and gene annotation were downloaded from Phytozome (http://www.phytozome.org; Goodstein et al. 2012). SyMAP v4.0 (Soderlund et al. 2011) with default parameters was used to identify syntenic blocks between the two genomes. The synteny visualization and analysis results can be found at http://cloud.rcc.uga.edu/symap/.

Expression analysis

To investigate candidate gene expression pattern, we performed infection kinetics as described in David et al. 2010 on JaloEEP558 with *C. lindemuthianum* strains 100 or C531 from Costa Rica, virulent on JaloEEP558 (Geffroy et al. 1999) and control plants were sprayed with water. Cotyledonary leaves from three different plants were harvested at 24, 48, 72, and 96 h after *C. lindemuthianum* infection as well as from the control plants and immediately frozen in liquid Nitrogen for mRNA isolation. Extractions of total RNA were carried out with the Nucleospin RNA plant Kit according to manufacturer's instructions (Macherey–Nagel, Düren, Germany). cDNA synthesis was performed using the Improm-II reverse-transcription system (Promega, France) with 1 µg of total RNA and oligo d(T) primers. Semi-quantitative reverse-transcription PCR (RT-PCR) was made using specific primers for each candidate genes and with Ubiquitin as internal control. Primer sequences are listed in Table S3. PCR products were analyzed on 2 % agarose gels containing ethidium bromide, run in 0.5× TBE buffer and visualized under UV light.

Results

Identification of *Co-x* flanking markers

The *Co-x* anthracnose R specificity, present in JaloEEP558 and conferring resistance against the strain 100 of *C. lindemuthianum*, was previously mapped at a distal position on LG B1, 12.4 cM from marker PROE8b using a set of 77 BAT93 × JaloEEP558 RILs (Geffroy et al. 2008). We mapped the PCR-based marker CV542014, previously described in the same distal position on LG B1 (Goncalves-Vidigal et al. 2011), at 2.7 cM from *Co-x* (Fig. 1a). To localize the *Co-x* region in the common bean genome, the two 25 bp and 21 bp CV542014 forward and reverse primer sequences, respectively (Supplementary Table S2), were used as queries in BLASTN analysis against G19833

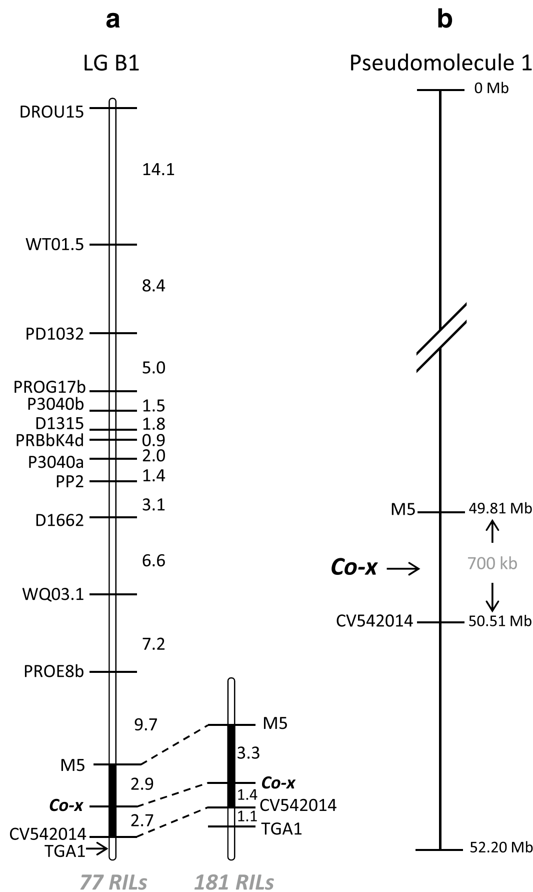


Fig. 1 Mapping of *Co-x* on LG B1 and identification of flanking markers. **a** Location of *Co-x* on LG B1 based on the 77 BAT93 × JaloEEP558 RILs population (Geffroy et al. 2008, 2000) and fine mapping of *Co-x* based on the 181 BAT93 × JaloEEP558 RILs population are represented on the *left* and on the *right*, respectively. Map distances are indicated between molecular markers and expressed in centiMorgans, estimated using the Kosambi mapping function. **b** Location of markers M5 and CV542014, flanking *Co-x*, on Pseudomolecule 1 of G19833. Marker names are indicated on the *left* and their coordinates on the *right*

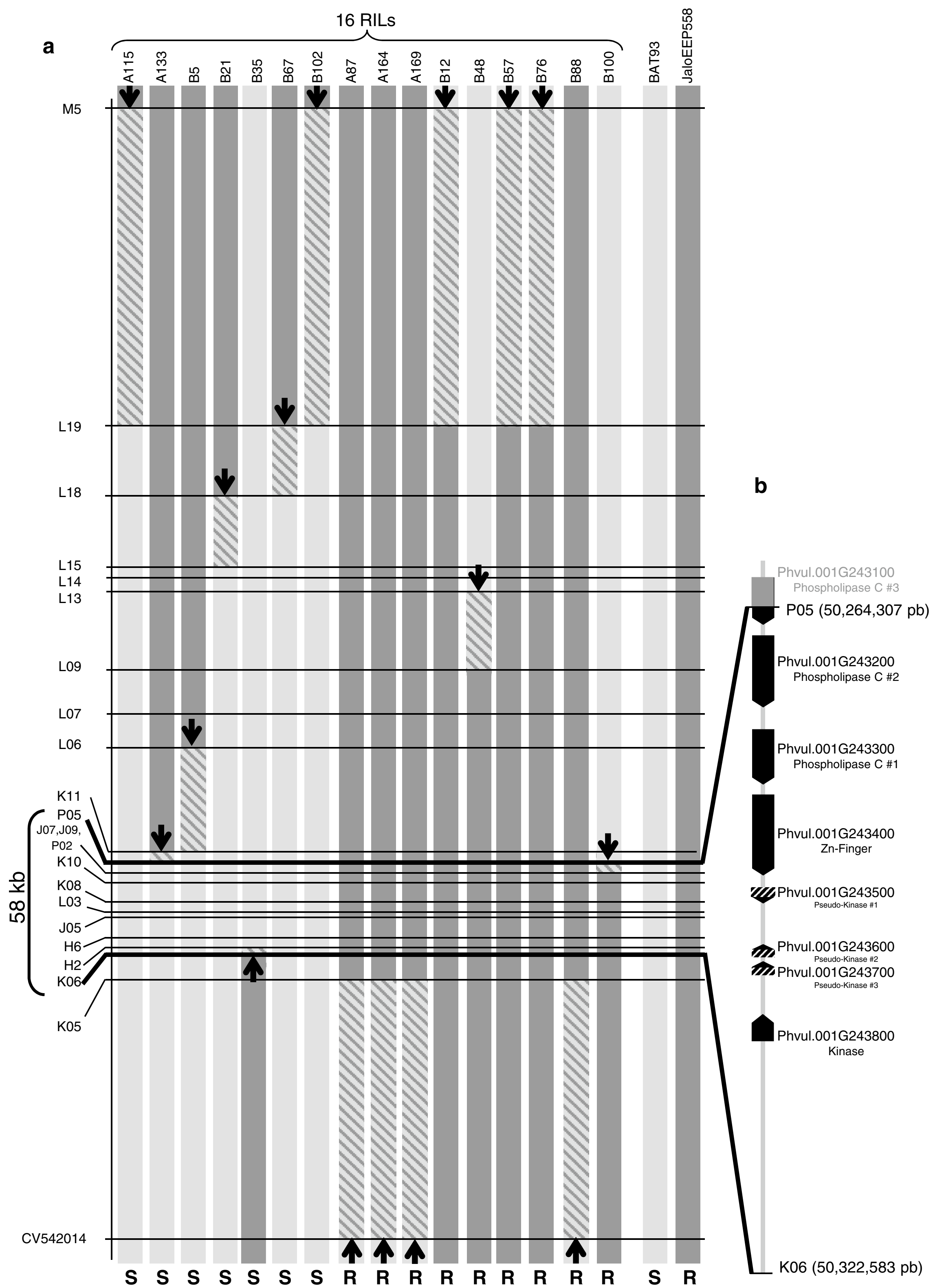
genome (www.phytozome.org). CV542014 forward and reverse primers are at nucleotide positions 50, 513, 853 and 50, 514, 196 of DNA sequence assembly corresponding to chromosome 1 (Pseudomolecule 1), respectively (Supplementary Table S2). To identify markers flanking *Co-x*, we generated three PCR-based markers, M3, M4 and M5, near CV542014, based on the G19833 genome sequence (Supplementary Table S2). M5 marker was found to be polymorphic between BAT93 and JaloEEP558 and mapped 2.9 cM from *Co-x* (Fig. 1a). In G19833 genome, markers M5 and CV542014 are at nucleotide positions 49, 805, 940–49, 806, 564 and 50, 513, 853–50, 514, 196 of Pseudomolecule 1, respectively (Supplemental Table S2). Therefore, *Co-x* is within a 700 kb region flanked by M5 and CV542014 (Fig. 1b).

Evaluation of strain 100 resistance on differential common bean cultivars and *Co-x* fine mapping

Pathogenicity tests were conducted to characterize *C. lindemuthianum* strain 100 on a set of differential common bean cultivars. Disease scoring revealed that strain 100 was highly virulent since it can overcome *Co-1*^{5}, *Co-2*, *Co-3*^{2}, *Co-4*, *Co-4*^{2}, *Co-4*^{3}, *Co-5*, *Co-5*^{2}, *Co-6*, *co-8*, *Co-9* and *Co-u* R genes (Supplementary Table S1). To map more precisely the resistance specificity *Co-x*, we increased the size of the mapping population using 104 additional BAT93 × JaloEEP558 RILs (Chen et al. 2010). The 104 additional RILs were inoculated with strain 100 and phenotypes were scored 6 and 7 days after inoculation. The ratio of susceptible (94 RILs) to resistant (87 RILs) of the total 181 RILs was evaluated using Chi-squared tests, and the observed segregation ratio fitted the expected 1:1 ratio of susceptible to resistant plants ($\chi^2 = 0.270$, $P = 0.6$) confirming that a single gene was segregating. On the total 181 RILs, *Co-x* was mapped between markers M5 and CV542014 at 3.3 and 1.4 cM, respectively (Fig. 1a), and we identified 16 RILs with a recombination event between these two flanking markers. Using the 700 kb genomic sequence of G19833, 52 PCR-based markers were generated between the flanking markers M5 and CV542014 (Supplementary Table S2 and Figure S1) and tested first on DNA of the parents BAT93 and JaloEEP558. In this way, 21 new polymorphic markers were generated and used to genotype the sixteen RILs with a recombination event between M5 and CV542014 markers. Graphical representations of the genotypes of these 16 key recombinant lines are presented in Fig. 2a. We observed cosegregation between the resistance phenotype and nine markers (P02, J07, J09, K10, K08, L03, J05, H6, and H2) (Fig. 2a). Therefore, *Co-x* is located in the region flanked by the two markers P05 and K06 which are at nucleotide positions 50, 264, 307–50, 265, 284 and 50, 320, 965–50, 322, 583 of G19833 Pseudomolecule 1, respectively. The interval containing *Co-x* was estimated to be 58 kb based on the common bean G19833 genome sequence (Fig. 2a).

Target region annotation and candidate gene prediction

Based on G19833 v1.0 JGI annotations (www.phytozome.org), eight predicted genes were located within the 58 kb region flanked by the markers P05 and K06 (Fig. 2b): three of these genes, Phvul.001G243100, Phvul.001G243200 and Phvul.001G243300, encode phosphoinositide-specific phospholipase C (PI-PLCs) proteins organized in a head to tail fashion, Phvul.001G243400 encodes a NF-X1 type zinc finger transcription factor, Phvul.001G243500, Phvul.001G243600 and Phvul.001G243700 encode truncated kinases, and Phvul.001G243800 encodes a full-length



kinase protein. Our manual annotation was consistent with JGI annotation. In the G19833 genome, two genes encoding NF-X1 type zinc finger transcription factors were identified using BLASTP searches, Phvul.001G243400, located in the *Co-x* 58 kb target region, and Phvul.002G331600 located on Pseudomolecule 2. Regarding kinases, all these predicted proteins shared sequence similarity with the *Arabidopsis thaliana* CRINKLY4 related

◀**Fig. 2** Fine mapping of *Co-x* and annotation of target region. **a** Physical map of *Co-x* region and graphical genotypes of RILs in which recombination occured between markers M5 and CV542014. *Light grey* and *dark grey bars* represent genomic regions derived from BAT93 and JaloEEP558, respectively. Phenotypes of resistance (R) and susceptibility (S) of RILs to *C. lindemuthianum* strain 100 are indicated below. For each RIL, a *black arrow* indicates the genetic interval carrying the inferred recombination breakpoint, represented by *light/dark grey* hatched motif. Location and name of polymorphic markers are indicated on the *left*. **b** Annotation of the *Co-x* 58 kb target region between markers P05 and K06 in G19833. Predicted candidate genes for *Co-x* resistance are indicated by *black* or *hatched arrows*, for full-length and truncated genes, respectively. Loci names according to www.phytozome.net and putative gene function are indicated on the *right*

3 (CRR3) protein. Phvul.001G243800 (2,421 bp) encodes a full-length CRINKLY4 related 3 protein, while Phvul.001G243500 (978 bp), Phvul.001G243600 (1,224 bp) and Phvul.001G243700 (1,236 bp) encode only the C-terminal part of the kinase. BLASTP analysis with *A. thaliana* CRINKLY4 related 3 (At3g55950) revealed that four full-length proteins are present in G19833 genome (Phvul.001G243800, Phvul.006G068400, Phvul.007G220700 and Phvul.008G223900). Analysis of the expression patterns of these candidate genes using a kinetic of infected or non-infected plants (Figure S3) revealed that all candidate genes were expressed. However, these expression analyses did not allow pointing out a particular candidate gene.

The PI-PLC family in common bean

Three out of the eight predicted candidate genes for *Co-x* encode PI-PLCs. In attempt to characterize the PI-PLC gene family in the common bean genome, we

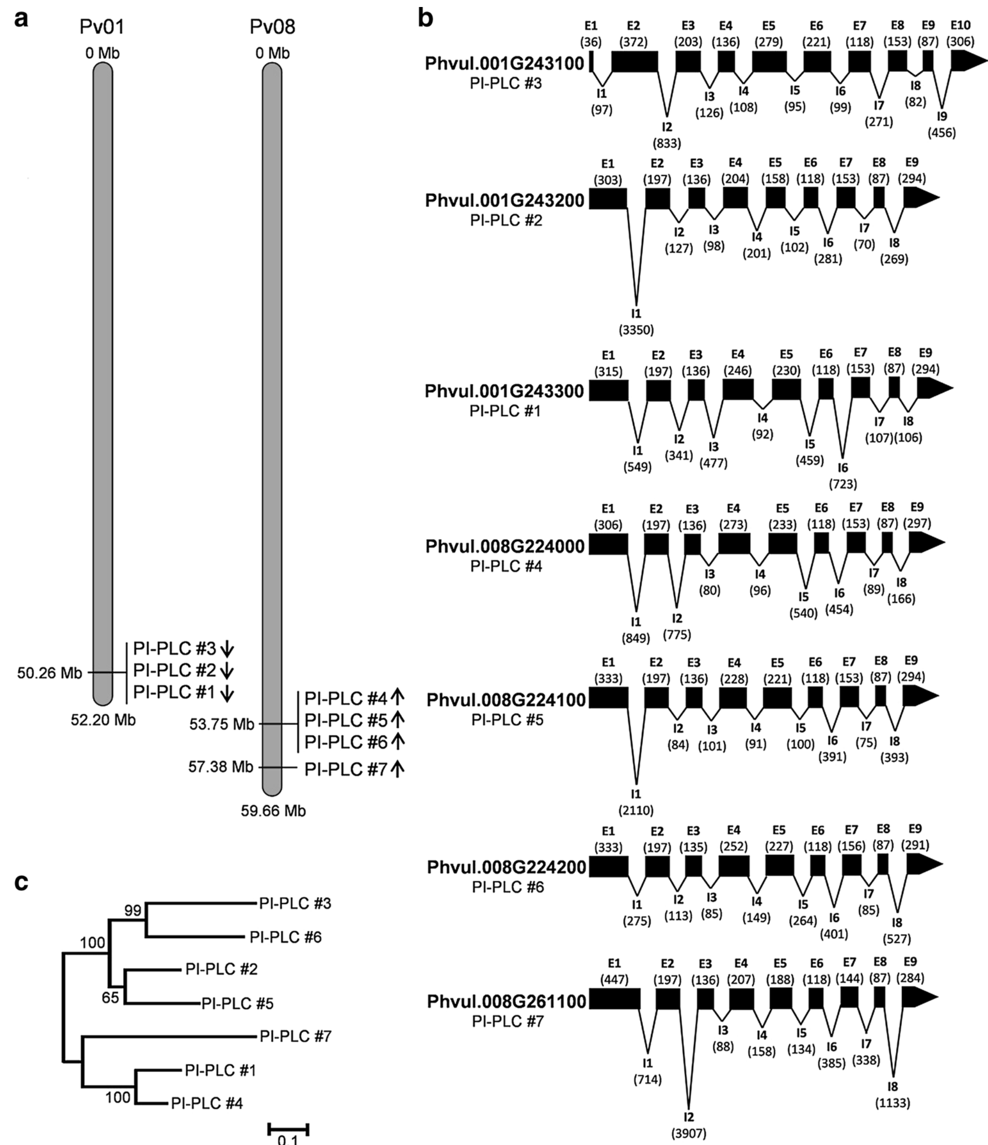


Fig. 3 Phosphoinositide-specific phospholipase C (PI-PLC) family in *Phaseolus vulgaris* G19833 genome (Phvul.001G243300, Phvul.001G243200, Phvul.001G243100, Phvul.008G224000, Phvul.008G224100, Phvul.008G224200 and Phvul.008G261100 numbered PI-PLC #1 to PI-PLC #7, respectively). **a** Genomic position of the seven PI-PLC coding genes on G19833 pseudomolecules. **b** Intron–exon structure of the seven PI-PLC coding genes. Exons (E1–E10) and introns (I1–I9) are numbered sequentially from left to right and are represented by *black boxes* and *black lines*, respectively. Size in base pairs of each exon and intron is indicated in *brackets*. **c** Maximum likelihood tree of *P. vulgaris* PI-PLC protein sequences

performed a BLASTP analysis using Phvul.001G243100, Phvul.001G243200 and Phvul.001G243300 predicted protein sequences as queries against common bean G19833 genome sequence (www.phytozome.org). This resulted in seven significant hits. In addition to the three PI-PLCs identified in the 58 kb *Co-x* target region (Phvul.001G243100, Phvul.001G243200 and Phvul.001G243300 referred to as PI-PLC #3, PI-PLC #2 and PI-PLC #1, respectively), we identified four other PI-PLCs at distal position of chromosome 8, Phvul.008G224000, Phvul.008G224100, Phvul.008G224200 and Phvul.008G261100 referred to as PI-PLC #4, PI-PLC #5, PI-PLC #6 and PI-PLC #7, respectively (Fig. 3a). As observed for PI-PLC #3, PI-PLC #2 and PI-PLC #1, three of these four additional PI-PLCs from chromosome 8 (PI-PLC #4, PI-PLC #5 and PI-PLC #6) are also organized in head to tail fashion in a small cluster, while PI-PLC #7 is 3.63 Mb away in a distal position of chromosome 8. Exon–intron structures of the seven different PI-PLC genes of *Phaseolus vulgaris* are quite similar (Fig. 3b). In all, the nucleic acid coding sequences were 1,911, 1,650, 1,776, 1,800, 1,767, 1,796, 1,808 pb for PI-PLC #3, #2, #1, #4, #5, #6 and #7, respectively. The number of exons varied from nine (PI-PLCs #2, #1, #4, #5, #6 and #7) to ten (PI-PLC #3) and exon sizes were highly conserved (Fig. 3b). The predicted amino acid sequences of these seven PI-PLC were aligned (Supplementary Figure S2). The derived maximum likelihood tree (Fig. 3c) shows strong relationships between PI-PLC #3 and PI-PLC #6, between PI-PLC #2 and PI-PLC #5 and between PI-PLC #1 and PI-PLC #4. Thus, in common bean genome, there are two related blocks each containing three PI-PLCs that are pairwise similar. These genes are predicted to encode typical plant PI-PLCs that belong to the PLCζ class consisting of a non-conserved N-terminal domain, followed by a conserved PI-PLC-X domain, a non-conserved spacer region, a conserved PI-PLC-Y and a conserved C2 or calcium-dependent lipid-binding (CaLB) domain at the C-terminus (Supplementary Figure S2) (Tasma et al. 2008).

Discussion

In this study, with the long-term objective of cloning *Co-x* in common bean, we fine-mapped this anthracnose *R* gene into an interval of 58 kb at the end of the long arm of chromosome 1. This study has been conducted in a favorable context. First, the availability of the common bean genome sequence greatly facilitated the development of precise PCR-based markers for fine mapping. Second, *Co-x* genetic mapping was carried out on a population of 181 RILs derived from a cross between the Mesoamerican breeding line BAT93 and the Andean landrace JaloEEP558. In addition to the fact that RILs populations are known to be as informative as F2 populations for small genetic distances (Fisher 1937), the size of the population combined with the high level of polymorphism between the Andean and the Mesoamerican parents associated with the numerous markers led to a good mapping resolution. Finally, the distal location of *Co-x* on chromosome 1 was also a positive point. Indeed, comparisons of genetic and physical distances in maize, soybean and common bean have all shown that these regions are highly recombinant as compared to centromeric regions (Gore et al. 2009; Schmutz et al. 2010, 2014).

The *Co-x* R gene is interesting for both applied and academic reasons. Regarding its agronomic interest, *Co-x* confers resistance to strain 100, an extremely virulent strain that in addition to being virulent to G 2333 (*Co-4*^{2}, *Co-5*^{2}) (Geffroy et al. 2008), a germplasm accession of Mexican origin and resistant to a broad range of *C. lindemuthianum* strains (Pastor-Corrales et al. 1994), also overcome most of the Mesoamerican anthracnose R genes (*Co-2*, *Co-3*^{2}, *Co-4*, *Co-4*^{3}, *Co-5*, *Co-6*, *co-8*, *Co-9* and *Co-u*) and an R gene of Andean origin (*Co-1*^{5}) (Supplementary Table S1). A combination of Andean and Mesoamerican R genes was proposed as a way of achieving durable resistance against anthracnose in common bean (Miklas et al. 2006). Indeed, adaptation of the *C. lindemuthianum* strains to plants of the same origin was observed in wild populations of common bean growing in the center of diversity of *Phaseolus vulgaris* as well as with domesticated beans (Geffroy et al. 1999). Consequently, *Co-x*, one of the scarce Andean R genes, appears to be of particular interest for such R gene pyramiding program. Combining resistance genes can be facilitated by the use of molecular markers linked to the relevant genes. The 55 locus-specific PCR-based markers generated in the present study (Supplementary Table S2) constitute the raw material for optimizing on the genotypes of interest markers that will be used in (*Co-x*) marker-assisted selection (MAS).

From a fundamental point of view, the *Co-x* gene is intriguing as none of the gene products within the *Co-x* interval resembles canonical plant immune receptors (Chisholm et al. 2006; Dangl and Jones 2001). We identified eight candidate genes belonging to three families: three phosphoinositide-specific phospholipase C (PI-PLCs) proteins, a NF-X1 type zinc finger transcription factor and four kinase proteins. Therefore, *Co-x* appears to be an atypical R gene, although these candidate genes are not outliers. Firstly, in animal cells, phospholipid-based signal transduction is a common mechanism for relaying extracellular signals perceived by transmembrane receptors (Berridge and Irvine 1989). In plants, PI-PLCs have been implicated in abiotic (Kim et al. 2004; Kocourkova et al. 2011; Tasma et al. 2008) and biotic (Chou et al.

2004; Vossen et al. 2010; Oh and Martin 2011) stress signal transductions. Regarding biotic stresses, a study on tomato showed that some PLCs are required for efficient plant defense responses against different pathogens including the fungi *Cladosporium fulvum* and *Verticillium dahliae* as well as the bacteria *Pseudomonas syringae* (Vossen et al. 2010; Oh and Martin 2011). Secondly, NF-X1 type zinc finger transcription factors also appear to be related to stress responses. As we identified in this study for common bean, two *NFX1*-like genes were commonly identified in plant genomes such as *AtNFXL1* and *AtNFXL2* in *Arabidopsis thaliana*. *AtNFXL1* is transcriptionally regulated under abiotic and biotic stress conditions such as osmotic and salt stresses (Lisso et al. 2006), heat stress (Larkindale and Vierling 2008), application of the T-2 mycotoxin (Asano et al. 2008), *Botrytis cinerea* and *Pseudomonas syringae* infections (Arabidopsis eFP Browser; http://bar.utoronto.ca/). Moreover, a *atnfxl1* knock-out mutant displayed an enhanced resistance to the pathogen Pst DC3000 (Asano et al. 2008). Interestingly, AtNFXL2 potentially plays an antagonistic role in stress responses (Lisso et al. 2006). Thirdly, kinases are implicated in a wide range of biological processes including disease resistance response. For instance, in tomato, the *Pto* gene, encoding an intracellular Ser/Thr protein kinase was previously identified as R gene against *Pseudomonas syringae* (Martin et al. 1993). More recent studies positioned Pto as the virulence target of pathogen effectors proteins of *P. syringae*, which in concert with the NB-LRR protein Prf, activates resistance response (Pedley and Martin 2003; Mucyn et al. 2006, 2009). Another example comes from wheat, where *Yr36* encodes a kinase-START conferring partial resistance to stripe rust (Fu et al. 2009). Furthermore, even if most of the R genes identified to date encode NB-LRR proteins, several exceptions have been reported as for instance, *mlo* in barley (Buschges et al. 1997; Devoto et al. 2003, 1999), *RPW8* in *Arabidopsis* (Xiao et al. 2001) or *Lr34* in wheat (Krattinger et al. 2009). For each of the aforementioned examples, the atypical R gene encodes a plasma membrane-associated protein suggesting a strategic location to early recognize and resist to pathogens. By analogy with animals, due to their function in transduction of extracellular signals perceived by transmembrane receptors, PI-PLCs appears as good *Co-x* candidate genes, although the NF-X1 type zinc finger transcription factor and kinases cannot be discarded.

Even though no genes encoding NB-LRR proteins have been identified in the 700 kb defined by markers M5 and CV542014 in G19833 sequence, this genomic region in the vicinity of *Co-x* locus is particularly rich in R genes effective against various pathogens. Indeed, resistances against rust (Jung et al. 1998; Park et al. 1999), angular leaf spot (Goncalves-Vidigal et al. 2011) and anthracnose (Geffroy et al. 2008) have been mapped near *Co-x* and an allelic series containing *Co-1*, *Co-1*^{2}, *Co-1*^{3} (Melotto and Kelly 2000), *Co-1*^{4} (Goncalves-Vidigal et al. 2011), *Co-1*^{5} (Goncalves-Vidigal and Kelly 2006) has been described. Intriguingly, all these resistance genes have been identified in Andean common bean genotypes, while other resistance clusters such as the *B4* cluster located at the end of chromosome 4 short arm contain both Mesoamerican and Andean R specificities (David et al. 2009; Geffroy et al. 1999).

Our results reveal that *Co-x* is not a member of the *Co-1* allelic series (Melotto and Kelly 2000; Goncalves-Vidigal et al. 2011; Goncalves-Vidigal and Kelly 2006). Indeed, *Co-1*^{4} and *Phg-1* R genes are located between markers CV542014 and TGA1 (Goncalves-Vidigal et al. 2011) while *Co-x* is located between P05 and K06 markers (Fig. 4b). Based on the position of markers CV542014 and K06, *Co-1*^{4} and *Phg-1* are separated by at least 190 kb from *Co-x* (Fig. 4b).

Common bean and soybean genome are known to have fractioned blocks of synteny (McClean et al. 2010). We found that *Co-x* and *Co-1*^{4} belong to a syntenic block located at the end of soybean chromosomes 11 (from 35.1 to 39.17 Mb) and 18 (from 3.67 to 0 Mb) (Fig. 4a). Interestingly, a soybean cyst nematode resistance gene (*rhg1*) has been mapped at the end of chromosome Gm18 in this syntenic region (Ruben et al. 2006). Recently, the *rhg1-b* allele from soybean line PI 88788 has been characterized and located between 1.63 and 1.65 Mb of Gm18 in the sequenced genotype Williams 82 (*Rhg1* in Fig. 4b) (Cook et al. 2012; Kim et al. 2010). Microsynteny analysis between common bean and soybean reveals that the block of synteny containing *Co-x* and *Co-1*^{4} in common bean and *Rhg1* in soybean are highly collinear with genes conserved in order and orientation (Fig. 4b). This result is in agreement with previous analysis showing extensive synteny in this region between soybean and *Medicago truncatula* (Mudge et al. 2005). In conclusion, this microsynteny analysis suggests that *Co-x* and *Rhg1* loci, although located in the same block of synteny, are located at distinct loci, separated by nearly 310 kb (Fig. 4b).

Our results point out a new complex disease resistance cluster in common bean that seems to be molecularly atypical. The emergence of an annotated genome sequence of G19833 greatly facilitated this analysis even though *Co-x* is present in JaloEEP558. Our current efforts are focused on recovering the 58 kb target sequence in JaloEEP558 before proceeding to functional validation using virus-induced gene silencing (VIGS) with a BPMV-derived VIGS vector (Zhang et al. 2010; Pflieger et al. 2013). All *Co-x* candidate genes belong to a small gene family which should facilitate the specific silencing of each of them.

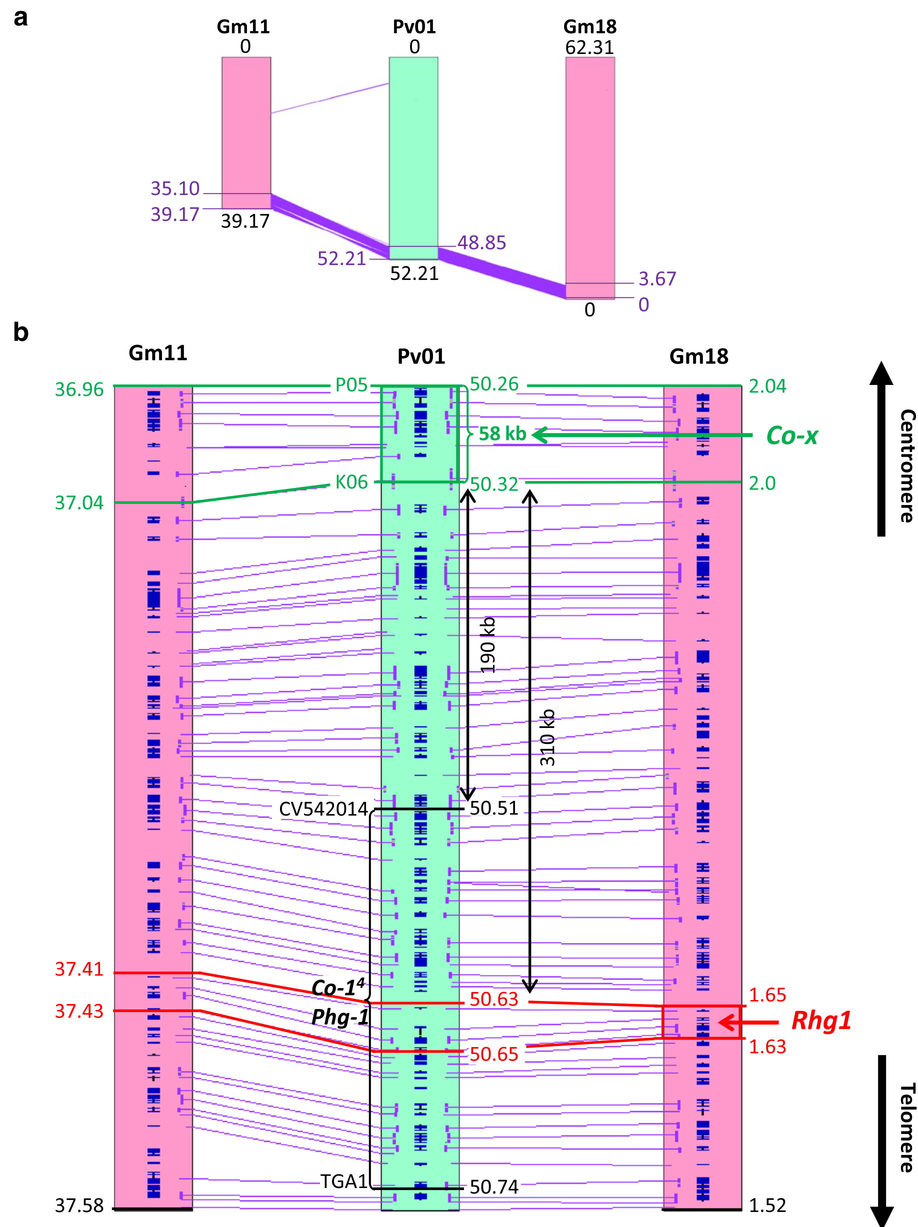


Fig. 4 Synteny analysis between the *Co-x* region of *Phaseolus vulgaris* on chromosome 01 (Pv01) and the corresponding regions in *Glycine max* chromosomes 11 and 18 (Gm11 and Gm18) generated with SyMAP v4.0 (Soderlund et al. 2011) (http://cloud.rcc.uga.edu/symap/). *Vertical light green* and *pink rectangles* represent common bean and soybean chromosome sequences, respectively. **a** Full chromosome view. Coordinates of the start (5′) and end (3′) of Gm11 and Pv01 are indicated in *black* in Mb above and below each chromosome, respectively. Orientation of Gm18 has been reversed, and start and end coordinates are indicated below and above the chromosome, respectively. *Purple areas* indicate the two soybean homeologous regions of Gm11 and Gm18, and the orthologous common bean region of Pv01 defined by anchors. Coordinates are indicated in *purple* in Mb on each side of each areas. **b** Zoomed view of a part of Gm11, Pv01 and Gm18 syntenic blocks containing resistance loci *Co-x* (from common bean, this study), *Co-1*^{4} and *Phg-1* (from common bean, Goncalves-Vidigal et al. 2011) and *Rhg1* (from soybean, Gm18, Cook et al. 2012; Kim et al. 2010). *Dark blue rectangles* are gene exons and *purple rectangles* connected by *purple lines* are anchors. *Co-x* target region is *boxed in green* on Pv01 and *transverse green lines* indicate the orthologous regions in Gm11 and Gm18. Coordinates in Mb of these regions on each chromosome are written in *green*. *Rhg1* locus is *boxed in red* on Gm18 and *transverse red lines* indicate the homeologous region in Gm11 and the orthologous regions in Pv01. Coordinates in Mb of these regions on each chromosome are written in *red* (color figure online)

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All experiments described in this manuscript comply with the current laws of the country in which they were performed.

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