

The *Co-4* locus on chromosome Pv08 contains a unique cluster of 18 *COK-4* genes and is regulated by immune response in common bean

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Abstract

Key message The common bean locus *Co-4*, traditionally referred to as an anthracnose-resistant gene, contains a cluster of predicted receptor-like kinases (*COK-4* and CrRLK1-like), and at least two of these kinases are co-regulated with the plant's basal immunity.

Abstract Genetic resistance to anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. and Magnus) Briosi and Cavara, is conferred by major loci throughout the *Phaseolus vulgaris* genome, named *Co*. The complex *Co-4* locus was previously reported to have several copies of the *COK-4* gene that is predicted to code for a receptor-like kinase (RLK). In general, plant RLKs are involved in pathogen perception and signal transduction; however, the molecular function of *COK-4* remains elusive. Using newly identified molecular markers (PvTA25 and PvSNP_{COK-4}), the SAS13 marker, *COK-4* sequences and phylogeny, and the recently released bean genome sequence, we determined the most probable boundaries of the *Co-4* locus: a 325-Kbp region on chromosome Pv08.

Out of the 49 predicted transcripts in that region, 24 encode for putative RLKs (including 18 *COK-4* copies) with high similarity to members of the *Catharanthus roseus* RLK1-like (CrRLK1L) protein family from different plant species, including the well-described FERONIA (FER) and ANXUR. We also determined that two RLK-coding genes in the *Co-4* locus (*COK-4-3* and *FER-like*) are transcriptionally regulated when bean plants are challenged with the flg22 peptide, a commonly used elicitor of plant immunity, or the bacterium *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight. While *COK-4-3* is activated during immune response, *FER-like* is downregulated suggesting that these genes could play a role in plant responses to biotic stress. These results highlight the importance of dissecting the regulation and molecular function of individual genes within each locus, traditionally referred to as resistance gene based on genetic segregation analysis.

Introduction

Plants have the innate ability to recognize conserved microbial molecular patterns and establish immune responses that can be triggered by a broad range of pathogens or highly specific to a particular pathogen. These responses can be addressed in two major layers of plant immunity: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006; Spoel and Dong 2012). PTI is induced by perception of PAMPs through pattern-recognition receptors (PRRs) located at the plant cell surface, and ETI is mediated by resistance (R) genes leading to hypersensitive response (HR). All kinds of phytopathogens can potentially activate PTI and/or ETI (Thomma et al. 2011), which may result in systemic plant responses such

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as induced systemic resistance (ISR) or systemic-acquired resistance (SAR). These immune responses involve intricate metabolic pathways mediated by several plant hormones, such as jasmonic acid (JA) and salicylic acid (SA) (Thomma et al. 2011).

Among many pathosystems used to study the molecular processes involved in plant–pathogen interaction, *Colletotrichum* species have long served as models for hemibiotrophic fungal pathogens (O’Connell et al. 2012), being used in early studies to elucidate the role of phytoalexins in common bean (*Phaseolus vulgaris* L.) resistance to the fungus (Kuć 1982). Besides its scientific importance, common bean is also the most economically important species of the genus *Phaseolus* and the primary dietary protein source for several populations, mainly in developing countries (Broughton et al. 2003). *Colletotrichum lindemuthianum* (Sacc. and Magnus) Briosi & Cavara is the causal agent of anthracnose in common bean, one of the most serious diseases in this crop throughout the world—not only because of its seed-borne nature, but also because of the great variability of this pathogen (Melotto et al. 2000). This disease is responsible for great losses of common bean yield (up to 100 %) and, therefore, it is one of the longest studied diseases of this crop (Kelly and Vallejo 2004; Singh and Schwartz 2010; Ferreira et al. 2013).

Understanding resistance against anthracnose is one of the main goals in common bean breeding programs as genetic resistance is the most-efficient and environmentally friendly control of crop diseases (Dodds and Rathjen 2010). Until now, 14 single anthracnose resistance loci (*Co-1* to *Co-14*) and 11 quantitative trait loci (QTL) were discovered in the common bean genome, out of which, 12 loci were identified in bean genotypes of Mesoamerican origin, and nine were identified in genotypes of Andean origin (Kelly and Vallejo 2004; Ferreira et al. 2013). The *Co-4* locus, first described in the genotype TO (Fouilloux 1979; Awale and Kelly 2001), confers resistance against several races of *C. lindemuthianum* (Balardin and Kelly 1998). A second allele, *Co-4*², was identified in the resistant differential cultivar G2333 that possesses a combination of three independent resistance loci, *Co-4*², *Co-5*, and *Co-7* (Young et al. 1998). The single dominant *Co-4*² allele provides greater resistance than the original *Co-4*, and it is recognized among the broadest-based resistance genes described in common bean (Balardin and Kelly 1998; Silverio et al. 2002). The molecular structure of the different alleles of *Co-4* remains to be determined.

The genomic structure of the *Co-4* locus has been defined using genetics and genomics tools. Sequencing of the bacterial artificial chromosome BAC 78L₁₇ (Vanhouten and MacKenzie 1999), which was identified with the SAS13 molecular marker (Young et al. 1998), revealed that the *Co-4* locus contains several putative orthologs of

Pto-like kinase genes, named *COK-4* (Melotto and Kelly 2001; Melotto et al. 2004). In silico analysis suggested that *COK-4* are the members of the receptor-like kinase (RLK) family that encode proteins with a superfamily kinase domain, including AT-binding and transmembrane domains (Melotto and Kelly 2001).

Receptor-like kinase (RLKs) are important PRRs that play a role in self- and nonself-recognition, including the perception of hormones (Shiu and Bleeker 2001), PAMPs, and pathogen effectors. Several RLK proteins involved in plant immunity have been identified, including Xa21 (Song et al. 1995), Pto (Sessa and Martin 2000), FLAGELLIN SENSING 2 (FLS2) (Chinchilla et al. 2006), BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) (Chinchilla et al. 2007), among others. FLS2 is one of the well-studied RLKs (Zipfel et al. 2004), which is involved in PTI through the perception of the bacterial PAMP flagellin, acting together with BAK1, to activate downstream immune responses (Chinchilla et al. 2007). Thus, mounting evidence suggests that RLKs are part of basal plant immunity against fungal and bacterial pathogens.

Owing to its similarity to RLKs, we reasoned that *COK-4* could be regulated by PAMPs and play a role in basal immunity against other phytopathogens in addition to *C. lindemuthianum*. We first defined the bean genomic region containing *Co-4* using genetic and genomic analyses; the locus is now placed in a 325-Kbp region close to the telomere of the Pv08 chromosome. Out of the 24 RLK-coding genes at the *Co-4* locus, 18 showed high nucleotide sequence similarity to the originally identified *COK-4* from the bean genotype SEL 1308 (Melotto and Kelly 2001). Functional analysis of two kinases in this locus (referred to as *COK-4-3* and *FER-like*) revealed that they are regulated upon leaf treatment with the PAMP flg22 and infection with *Pseudomonas syringae* pv. *phaseolicola* (Pph). These findings suggest that the *Co-4* locus not only confers resistance against the anthracnose fungus, but it is also involved in the early stages of PTI in common bean.

Materials and methods

Mapping population and *C. lindemuthianum* pathogenesis assay

The common bean breeding line SEL 1308 was used as the source of the *Co-4*² allele in a cross with Black Magic, an anthracnose-susceptible black bean cultivar. Hybrid seeds were advanced to the F₂ generation, and 98 randomly selected F₂ individuals were used as a mapping population (Melotto and Kelly 2001). Plants were grown in controlled environment at 22 °C, 80 % relative humidity, and 16 h of

daylight. Ten-day old seedlings were spray inoculated with race 73 of *C. lindemuthianum*, which is avirulent on bean plants carrying the *Co-4*² allele (Young et al. 1998). Inoculum preparation, inoculation methods, and disease symptoms evaluation were conducted as described by Young and Kelly (1996).

Molecular marker development

Simple sequence repeat (SSR) markers were developed based on the DNA sequence of the BAC 78L₁₇ that was mapped to the *Co-4* locus (Melotto et al. 2004). SSRs were searched in the BAC sequence using the SSRIT software (<http://www.gramene.org/db/markers/ssrtool>; Temnykh et al. 2001). Among the SSR markers (Table S1), PvTA25 showed polymorphism between the SEL 1308 and Black Magic and was used to genotype the F₂-segregating population. The PCR reaction (25 µl) consisted of 1.5 mM MgCl₂, 1 × enzyme buffer, 200 µM dNTP, 1U *Taq* polymerase (Promega, Madison, WI), 50 ng DNA, and 25 ng of each primer (Table S2). The PCR cycle was 2 min at 94 °C; plus 13 cycles of 30 s at 94 °C; 30 s at 70 °C (with 1 °C decrease per cycle); 2 min at 72 °C; and 20 cycles of 30 s at 94 °C; 30 s at 57 °C; and 2 min at 72 °C, followed by a final extension cycle of 7 min at 72 °C. PCR products were resolved in 6 % polyacrylamide gel fixed in 1 % acetic acid and 10 % ethanol solution for 10 min, followed by a wash with distilled water for 1 min. The gel was soaked in 1.5 % nitric acid for 3 min and rinsed with distilled water for 1 min. The gel was stained with 0.2 % silver nitrate for 20 min followed by a final wash with distilled water for 30 s each. Developing was conducted with a solution of sodium carbonate (30 g/L) and 37 % formaldehyde (0.54 ml/L). Blocking was performed with 5 % acetic acid.

Amplified fragment length polymorphism (AFLP) markers were identified by means of bulk segregant analysis (BSA; Michelmore et al. 1991). DNA from F₂ individuals were bulked in resistant and susceptible pools based on the anthracnose response of each individual. Bulk DNA was digested with *EcoRI* and *MseI* restriction enzymes, followed by adaptor ligation, and pre-selective amplification using adaptor-specific primers containing one additional base (Table S2). Selective amplification was performed with primers containing two more random bases. PCR conditions were exactly the same as described by Hazen et al. (2002). Amplicons were resolved in 6 % polyacrylamide gel following the same protocol described for the SSR analysis. Amplicons showing polymorphism between the parents and the bulks were used to genotype the F₂ individuals to estimate genetic distance between polymorphic markers and the *Co-4*² allele.

Single nucleotide polymorphism (SNP) markers were developed to assay polymorphisms observed in the *COK-4*

open reading frame (ORF) sequences of contrasting bean genotypes (Melotto and Kelly 2001). Primers were designed to detect both parental alleles of the *COK-4* gene in the F₂-mapping population. One forward primer was designed to anneal with both homologs, and two reverse primers were designed to specifically anneal to one of each homolog (Table S2). The PCR was optimized to amplify both homologs in the same reaction to detect heterozygous genotypes. The reaction consisted of 1 × enzyme buffer, 3.5 mM MgCl₂, 200 µM dNTP, 1.5 U *Taq* DNA polymerase (Gibco), 15 ng of forward primer, 15 ng of the Black Magic homolog reverse primer, and 30 ng of the SEL 1308 reverse primer in a 30 µl reaction. The thermocycling profile consisted of one cycle of 94 °C for 4 min, 30 cycles of 94 °C for 10 s, 70 °C for 30 s, and 72 °C for 2 min, followed by an extension cycle of 72 °C for 7 min.

Genetic linkage analysis

In addition to the newly developed markers, individual F₂ plants from the Black Magic × SEL 1308 population were also screened with the previously found SCAR markers linked to the *Co-4* locus: SAS13 (Young et al. 1998), SBB14, and SH18 (Awale and Kelly 2001). The amplification conditions were the same as described in the respective publications for each marker. Statistical probability for single locus inheritance of molecular markers in the F₂ population was estimated with the Chi-square test. A linkage map around the *Co-4* locus was obtained using the MAPMAKER 3.0b software (Lander et al. 1987) with thresholds of 3.0 LOD score value and 37.5 centimorgan (cM) of maximum genetic distance. Loci were ordered using the “order” command, and the final order was tested using the “ripple” command with a window of six markers. Finally, multipoint distance estimates were obtained using the “map” command, with the cM distances among markers, and the *Co-4* locus being calculated by the Kosambi mapping function. The linkage map diagram was created using the MapChart 2.2 program (Voorrips 2002).

Physical localization of DNA markers and sequence analysis

All molecular markers with known sequence and tight linkage to *Co-4* were used to define the physical location of this locus in the G19833 reference bean genome sequence v1.0 available at Phytozome (<http://www.phytozome.net/>; Schmutz et al. 2014). The markers used were: PvTA25 and PvSNP_{COK-4} from this study; SAS13 (a 978-bp sequence obtained from the genotype SEL 1308; Melotto et al. 2004); SH18 and SBB14 kindly provided by James Kelly and Halima Awale; and SCARY20 (phaseolusgenes ID 548) and SCARC08 (phaseolusgenes ID 334) (Queiroz

et al. 2004; <http://phaseolusgenes.bioinformatics.ucdavis.edu/>). The SEL 1308 *COK-4* ORF (NCBI accession number GI:9796477; <http://blast.ncbi.nlm.nih.gov/>) and the whole sequence of the BAC 78L₁₇ (NCBI accession number GI:38194906) were also aligned to the bean genome. Alignment between DNA marker and the reference genome sequences was performed using BLASTN with default parameters (E-value $<1 \times 10^{-5}$ and identity ≥ 70 %) to define marker location. In addition, pairwise alignments between each marker and the *Co-4* region were performed using the BLASTN tool available at NCBI (bl2seq; Tatusova and Madden 1999) to refine the E-value for each marker. All the predicted transcripts in the *Co-4* locus were obtained from the Phytozome website, and the putative functions of the genes were inferred based on Pfam annotations also available through the Phytozome database.

Phylogenetic analysis

First, the top 100 hits of putative paralogs of the COK-4 kinase in common bean were identified using the predicted COK-4 protein from the bean line SEL1308 (COK-4_SEL1308) as query against the common bean proteome database available at Phytozome (BLASTP, threshold E-value $\leq 1 \times 10^{-20}$ and identity >30 %). These 100 sequences and the SEL 1308 COK-4 were aligned with CLUSTALW as part of the MEGA 5.05 software (Tamura et al. 2011). The conserved catalytic tyrosine kinase domain of these predicted bean kinases was identified by searching the COK-4 protein sequence against the NCBI protein-conserved domain database (CDD) (Marchler-Bauer et al. 2013). The phylogenetic tree was created by means of MEGA 5.05 using the maximum parsimony method, and bootstrap support values were obtained from 1000 replications.

The phylogeny analysis described above was also performed with the top 100 hits of COK-4_SEL1308 against the non-redundant (nr) protein database of all species currently available at NCBI (BLASTP, threshold E-value $\leq 1 \times 10^{-20}$ and identity >30 %).

Pathogenesis assay

Seeds of the bean genotypes G2333 and the halo blight susceptible control Beluga were germinated on filter paper in a growth chamber at 28 °C and 12-h photoperiod for 3 days. Seedling were transplanted to 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite and grown in controlled environmental chambers at 28 °C, 60 ± 5 % relative humidity, and a 12-h photoperiod under light intensity of $100 \mu\text{mol}/\text{m}^2/\text{s}$. *Pseudomonas syringae* pv. *phaseolicola* (Burkn.) Downs (Pph) strain NPS3121 was grown in

low-salt Luria–Bertani medium (Katagiri et al. 2002) at 30 °C supplemented with 100 $\mu\text{g}/\text{ml}$ rifampicin. Young, fully expanded primary leaves were dip inoculated into 1×10^8 CFU/ml aqueous suspension containing 0.03 % Silwet L-77 (Lehle Seeds Co., Round Rock, TX). Inoculum preparation and bacterial population counts in the leaf apoplast were performed as previously described (Katagiri et al. 2002). Statistical significance of the mean difference between the bean genotypes was detected with two-tailed Student's *t* test. Typical disease symptoms were observed and photographed at 7 days after inoculation.

Callose deposition assay

G2333 seeds were germinated and grown as described above. Fully expanded, first trifoliolate leaves were syringe infiltrated with 1 μM of flg22 (Alpha Diagnostics, Inc., Santa Monica, CA) or water. Infiltrated leaves were collected 12 and 24 h post-infiltration and incubated in 90 % ethanol at 37 °C in an orbital shaker (30 rpm). After the chlorophyll had been removed, leaves were rinsed in 50 % ethanol followed by a final rinse in water. Cleared leaves were stained with 0.1 μM aniline blue for 30 min and maintained in 50 % glycerol. Images (12–15 per sample) were captured by a Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Shinagawa-ku, Tokyo) equipped with DAPI filter (358-nm excitation and 461-nm emission) and a digital camera. Callose deposits were counted using the DotCount v1.2 software (Reuter 2012; <http://reuter.mit.edu/software/dotcount/>), using an image intensity threshold of 100 and dot sizes ranging from 5 to 500. Experiments were performed two times independently.

Gene expression analysis

Gene-specific primers were designed based on the common bean gene sequences from the Phytozome database (Table S3). The efficiency of each primer set was verified using a fivefold serial dilution of G2333 cDNA. Linear regression between the amounts of cDNA template and the corresponding C_T values were calculated for each primer set to obtain the correlation coefficient ($R^2 > 0.95$) according to Schmittgen and Livak (2008).

Fully expanded primary leaves of G2333 were dip inoculated with either 1×10^8 CFU/ml Pph suspension with 0.03 % Silwet or 0.03 % Silwet alone (mock inoculation). Leaves were collected at 6, 12, and 24 h post-inoculation (hpi). In addition, young first trifoliolate leaves were immersed in 5 μM flg22 or water control for 30 min. Leaves were maintained in high humidity inside a growth chamber set at 28 °C and 12-h photoperiod, and were collected 6, 12, or 24 h after flg22 treatment. Total RNA was extracted from leaves using the RNeasy Plant mini kit

(Qiagen, Valencia, CA) following the manufacturer's recommendations. The total RNA was quantified using a NanoDrop spectrophotometer (Thermo 367 Scientific, Rockford, IL).

Reverse transcription (RT) was performed using the Takara RNA PCR kit (Clontech, Mountain View, CA), 150 ng/ μ l of total RNA, and 0.125 μ M of oligo-dT primer, according to the manufacturer's protocol. RT reaction was carried out at 50 °C for 30 min and at 95 °C for 5 min. Quantitative PCR (qPCR) reactions were carried out using 1 μ l of cDNA (RT reaction above), 200 mM of each primer (Table S3), and iTaq Fast SYBR green supermix (BioRad, Hercules, CA) reagents in a final volume of 20 μ l. qPCR cycles consisted of one cycle of 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, followed by the dissociation-curve default parameters using the Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA). The gene expression levels of the treated samples relative to control samples were determined by means of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The *P. vulgaris* *INSULIN DEGRADING ENZYME* (*PvIDE*; Phvul.001G133200) gene (Borges et al. 2011) was used as the reference gene for the amount of RNA template across different reactions. The genes analyzed were *FLS2-like* (*LRR*) (Phvul.005G149200), *FLS2-like* (*RLK*) (Phvul.002G196200), *COK-4-3* (Phvul.008G026900), *FER-like* (Phvul.008G030800), *NB-LRR* (Phvul.008G031200),

and *FUL-like* (Phvul.008G027800). All experiments were performed in three biological replicates, and statistical analysis was conducted using the two-tailed Student's *t* test.

Results

Molecular markers define the genetic boundaries of the *Co-4* locus

Several markers closely linked to the anthracnose resistance *Co-4* locus of common bean have been identified (Kelly and Vallejo 2004; Ferreira et al. 2013); however, the genomic boundaries of this locus were still elusive. Thus, we sought to refine its genetic structure by saturating this locus with new molecular markers and determine the segregation ratios of all possible polymorphic markers using the SEL 1308 \times Black Magic F_2 population (Melotto and Kelly 2001). A SSR marker was developed based on the sequence of the previously identified clone (BAC 78L₁₇) that spans part of the complex *Co-4* locus (Melotto et al. 2004). A 149-bp TA-repeat marker, named PvTA25, showed a co-dominant polymorphism between the parents of the mapping population (SEL 1308 and Black Magic) as well as polymorphisms among bean lines carrying contrasting alleles at the *Co-4* locus (Fig. 1a). All three lines known

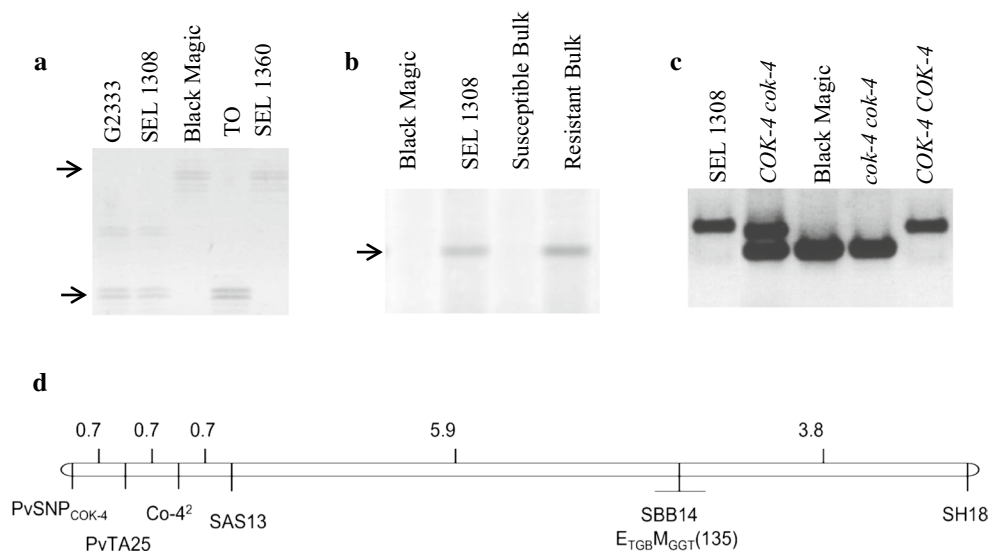


Fig. 1 Molecular marker banding pattern in resistant and susceptible genotypes of common bean. G2333, SEL 1308, and TO are homozygous anthracnose-resistant genotypes, Black Magic and SEL1360 are homozygous anthracnose-susceptible genotypes. **a**, **b** Silver-stained polyacrylamide gel showing polymorphisms (scored bands are indicated by the arrows) detected with the PvTA25 SSR primers (**a**) and the $E_{TGBM_GGT(135)}$ AFLP (**b**) markers. **c** Ethidium bromide-stained agarose gel showing PCR-amplified DNA fragments obtained with the $PvSNP_{COK-4}$ marker (700-bp lower band and 1000-bp upper

band); legends on top of the lanes indicate the genotype of F_2 individuals from the cross between SEL 1308 and Black Magic. **d** Predicted genetic distance among the markers linked to the *Co-4* locus. The numbers on the top are estimated distances between molecular markers in centiMorgans (cM) calculated with the MapMaker software considering LOD score >3 as threshold. The linkage map diagram was created using the MapChart 2.2 program (Voorrips 2002) and the scale was set as 10 mm/cM

Table 1 Segregation analysis of molecular markers linked to the *Co-4*² allele using an F₂ mapping population derived from the SEL 1308 × Black Magic genetic cross

Locus	Expected segregation ratio	Observed frequency	<i>P</i> ^a	Marker type
PvTA25	1:2:1	19:45:24	0.74	SSR
E _{TGC} M _{GGT} (135)	3:1	69:28	0.38	AFLP
SAS13	3:1	70:27	0.52	SCAR
SBB14	1:2:1	17:52:28	0.22	SCAR
SH18	3:1	65:31	0.10	SCAR
PvSNP _{COK-4}	1:2:1	200:426:223	0.53	SNP
<i>Co-4</i> ² / <i>co-4</i> ²	3:1	626:223	0.39	–

^a *P* = statistical probability calculated with the Chi-square test

to carry the *Co-4*-resistant allele (G2333, SEL 1308, and TO) showed the same PvTA25 marker allele, while the susceptible genotypes Black Magic and SEL 1360 shared a PvTA25 DNA fragment of higher molecular weight (Fig. 1a). A dominant single locus AFLP marker, named E_{TGC}M_{GGT}(135), was also identified to be linked to the *Co-4* locus, with the presence or the absence of a 135-bp PCR amplicon in the resistant and susceptible genotypes, respectively (Fig. 1b). Finally, an allele-specific SNP marker, named PvSNP_{COK-4}, was developed by aligning the *COK-4* sequences from SEL 1308 and Black Magic. Segregation analysis of this marker revealed its co-dominant nature, in which heterozygous *COK-4* individuals carried both the 700-bp susceptible and 1000-bp resistance alleles (Fig. 1c).

The PvTA25, AFLP E_{TGC}M_{GGT}(135), and PvSNP_{COK-4} markers, as well as the previously identified SAS13 molecular marker, segregated as a single locus in our mapping population as determined by Chi-square statistical analysis (Table 1) and were closely linked to *Co-4* as determined by linkage mapping analysis (Fig. 1d). The previously identified SBB14 and SH18 markers (Awale and Kelly 2001) were also tested in our segregating population, and they showed a low Chi-square *P* value, indicating that they might not be a single locus (Table 1); nonetheless, both were found to be linked to *Co-4* based on linkage mapping analysis (Fig. 1d). The genetic order of all markers around the *Co-4* locus was estimated using the genotypic and phenotypic data of F₂ individuals. SAS13, PvTA25, and PvSNP_{COK-4} were the closest markers to *Co-4*, all within 0.7 cM from each other, whereas E_{TGC}M_{GGT}(135) and SBB14 markers mapped at 6.6 cM away from *Co-4*, and SH18 mapped at 10.4 cM away from *Co-4* (Fig. 1d).

Physical location of markers linked to *Co-4* in the bean genome

The farthest marker away from the *Co-4* locus, SH18, showed sequence similarity with many regions in

different chromosomes ($1 \times 10^{-149} \leq \text{E-value} \leq 1 \times 10^{-142}$), including Pv08 at the 54,381,505–54,382,406 region (Fig. 2). The markers SCARY20 and SCARC08, previously mapped at 1.2 and 7.8 cM away from the *Co-4* locus for the TO genotype, respectively (Queiroz et al. 2004), show the best alignment scores (BLASTN E-value = 0.0) on Pv08 at positions 28,034,637–28,034,904 and 7,414,123–7,415,017, respectively (Fig. 2). These markers also aligned with different regions on Pv08 as well as other bean chromosomes ($0.0 \leq \text{E-value} \leq 1 \times 10^{-134}$). SH18, SCARY20, and SCARC08 were located at genome regions with no predicted coding sequences. The SBB14 marker sequence, however, was found at the position 2,809,493–2,810,488 of Pv08 (BLASTN E-value = 0.0), approximately 240 Kbp away from the *Co-4* locus (Fig. 2), in the 5'UTR from the Phvul.008G033800, a predicted amylase gene, and does not have significant similarity to any other region of the bean genome.

Consistently, the tightly linked PvTA25, PvSNP_{COK-4}, and SAS13 marker sequences were also found at unique regions of the Pv08 chromosome (Fig. 2). SAS13 is located in the Phvu1.008G028500 gene (BLASTN E-value = 0.0), PvTA25 is 650 bp away from the Phvu1.008G029500 gene (BLASTN E-value = 4×10^{-40}), and the PvSNP_{COK-4} primers align within the Phvu1.008G028400 gene (BLASTN E-value = 1×10^{-7}), with a predicted DNA fragment of 724 bp, similar to the one amplified from Black Magic (Fig. 1C). None of these markers aligned at a different genomic location, confirming the single locus segregation data analysis (Table 1; Awale and Kelly 2001; Melotto and Kelly 2001).

Previously, the BAC 78L₁₇ has been physically located at the chromosome Pv08 as revealed by FISH analysis (Melotto et al. 2004). BLASTN analysis of the BAC 78L₁₇ against the common bean genome v1.0 (Schmutz et al. 2014) located this BAC in the 2,345,000–2,464,000 region of Pv08 (BLASTN E-value = 0.0). BLASTN alignment between the previously identified *COK-4* gene sequence from SEL 1308 (Melotto et al. 2004), and the bean genome revealed 20 significant hits on Pv08 (E-value $\leq 1 \times 10^{-64}$ and identity >70 %; Table 2), extending our previous description of the BAC 78L₁₇ region that contains 10 sequences with similarity to *COK-4* (Fig. 2). Out of the 20 hits, 18 lie on a genomic region with predicted transcripts encoding a tyrosine kinase domain (Table S4). The *COK-4* copies were numbered according to their order of location in the genome (Table 2). Outside the Pv08 chromosome, only one match to the SEL 1308 *COK-4* gene was found on chromosome Pv05 (Table 2), in a region with no predicted transcript. Furthermore, none of the genetic markers tightly linked to *Co-4* were found on chromosome Pv05; thus, we have not considered it as a possible location for this anthracnose-resistant locus. Thereby, we predict that

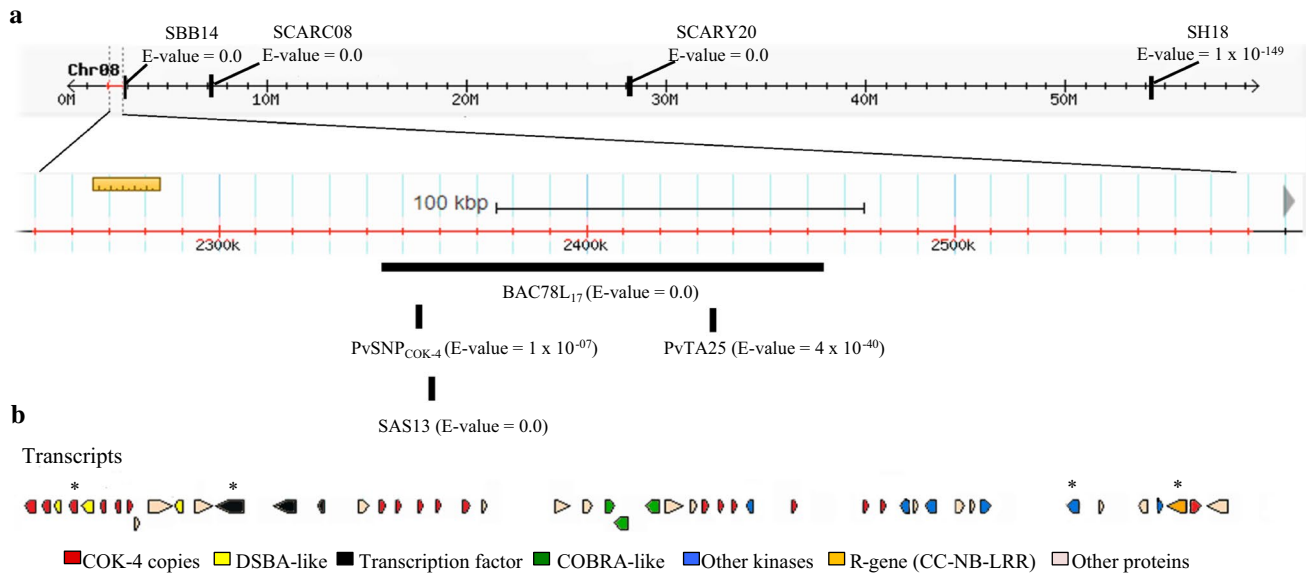


Fig. 2 Genomic boundaries and structure of the bean anthracnose resistance locus *Co-4*. **a** Genome browser representation of the 325 Kbp in the chromosome 8 (Chr08) spanning the *Co-4* locus that contains the markers SAS13, PvTA25, and PvSNP_{COK-4}, as well as the BAC 78L₁₇ sequences. Physical location was determined by BLASTN analysis (threshold E-value $\leq 1 \times 10^{-5}$) of the marker

sequences against the common bean genome v.1.0 (Schmutz et al. 2014; <http://www.phytozome.net/>). **b** Predicted transcripts in *Co-4* region are shown below their genomic positions in (a). Color codes as indicated in the legend below the figure, represent predicted gene functions. Asterisks above the transcript indicate genes that were analyzed by RT-qPCR

the *Co-4* locus is the most likely to be at the 325-Kbp region (Chr08:2,245,000–2,570,000) adjacent to the Pv08 telomere (Richard et al. 2013) based on genetic linkage (Fig. 1), the genomic location (Fig. 2) of the markers closely linked to *Co-4* (SAS13, PvTA25, and PvSNP_{COK-4}), and the presence of multiple potential *COK-4* paralogs in that region (Fig. 2; Table 2).

The *Co-4* locus is enriched with putative kinases of the CrRLK1L family

Once the 325-Kbp region on Pv08 (Chr08:2,245,000–2,570,000) was determined as the most likely one to contain the *Co-4* locus, we sought to characterize its gene content. Forty-nine transcripts were identified (Fig. 2 and Table S4) with the support of expression data obtained with RNA-seq and EST analyses (Phytozome). Function annotation of the transcripts revealed three putative transcription factors located next to each other in the chromosome (two SRF-type transcription factors and one Myb-like domain), three DSBA-like (disulfide oxidoreductase-like), three COBRA-like, one NB-LRR (Nucleotide Binding–Leucine-Rich Repeat) domain-encoding gene, eleven genes showing various putative functions, and four with unknown functions (Table S4). Twenty-four transcripts in the *Co-4* region are predicted to encode protein kinases with significant similarity to the predicted SEL

1308 *COK-4* protein (BLASTP E-value $\leq 2 \times 10^{-31}$; Table S5). Four of the *COK-4* gene copies showed the highest similarity (BLASTP E-values = 0.0) to the protein *COK-4* from SEL 1308: Phvul.008G028300 (identity = 81.5 %), Phvul.008G028400 (identity = 78.4 %), Phvul.008G028500 (identity = 83.4 %), and Phvul.008G028600 (identity = 84.0 %) (Table S5). All predicted kinases in the *Co-4* region showed significant similarity with members of the Arabidopsis CrRLK1L family: FERONIA (FER), ANXUR2 and AT5G39000 (Table S5). In addition, BLASTP analysis of *COK-4*_SEL1308 against the nonredundant database of NCBI showed high similarity with CrRLK1L family members from different plant species, with FER and ANXUR being also overrepresented (Table S6). Phylogenetic analysis of these proteins showed that *COK-4* forms a major clade with serine/threonine kinases from *Glycine max*, *Cicer arietinum*, *Lotus japonicus*, *Theobroma cacao*, and *Malus domestica*, as well as Pto-like proteins from three *Solanum* species and *Capsicum chinense* (Fig. 3).

Among the 24 putative kinases in the *Co-4* locus, 20 are predicted to encode a single kinase domain protein, and four are predicted to encode both a kinase and a lectin domain (Table S5). Lectin is an endoplasmic reticulum membrane-anchored domain, and is found in proteins of the CrRLK1L family (Kessler et al. 2010), among other protein families. Three of these proteins

Table 2 Common bean genome regions similar to the SEL 1308 *COK-4* gene

Common bean gene	<i>COK-4</i> copy ^a	E-value ^b	Identities	Gaps	Alignment scheme ^c
Phvul.008G026600	<i>COK-4-1</i>	1×10^{-149}	776/1098 (71%)	75/1098 (6%)	
Phvul.008G026700	<i>COK-4-2</i>	4×10^{-108}	433/581 (75%)	11/581 (1%)	
Phvul.008G026900	<i>COK-4-3</i>	7×10^{-104}	671/890 (75%)	28/890 (3%)	
Phvul.008G027100	<i>COK-4-4</i>	1×10^{-98}	666/887 (75%)	25/887 (3%)	
Phvul.008G027200	<i>COK-4-5</i>	1×10^{-142}	773/1097 (70%)	70/1097 (6%)	
Phvul.008G027300	<i>COK-4-6</i>	6×10^{-140}	757/1067 (71%)	45/1067 (4%)	
Phvul.008G028200	<i>COK-4-7</i>	7×10^{-128}	793/1049 (76%)	12/1049 (1%)	
Phvul.008G028300	<i>COK-4-8</i>	0.0	1038/1102 (94%)	5/1102 (0%)	
Phvul.008G028400	<i>COK-4-9</i>	0.0	1034/1100 (94%)	14/1100 (1%)	
Phvul.008G028500	<i>COK-4-10</i>	0.0	1049/1100 (95%)	3/1100 (0%)	
Phvul.008G028600	<i>COK-4-11</i>	0.0	1093/1111 (98%)	1/1111 (0%)	
Phvul.008G029500	<i>COK-4-12</i>	0.0	781/1061 (74%)	38/1061 (3%)	
Phvul.008G029600	<i>COK-4-13</i>	6×10^{-172}	754/1031 (73%)	50/1031 (4%)	
Phvul.008G029700	<i>COK-4-14</i>	2×10^{-165}	751/1035 (73%)	63/1035 (6%)	
Phvul.008G029900	<i>COK-4-15</i>	6×10^{-93}	403/550 (73%)	25/550 (4%)	
Phvul.008G030000	<i>COK-4-16</i>	3×10^{-169}	752/1031 (73%)	50/1031 (4%)	
Phvul.008G030100	<i>COK-4-17</i>	3×10^{-175}	756/1035 (73%)	59/1035 (5%)	
Phvul.008G031300	<i>COK-4-18</i>	5×10^{-64}	547/758 (72%)	36/758 (5%)	
Common bean region		E-value ^b	Identities	Gaps	
Chr08:2438870..2439364		4×10^{-68}	358/495 (72%)	12/495 (2%)	
Chr08:2450588..2451617		4×10^{-157}	748/1030 (73%)	40/1030 (4%)	
Chr05:20356383..20356071		7×10^{-47}	231/313 (74%)	1/313 (0%)	

^a Putative *COK-4* copies were named according to the gene order on Pv08, without implying functionality

^b BLASTN analysis was conducted using the *COK-4*_SEL1308 genomic sequence as query (1110 bp) against the common bean reference genome (Phytozome v1.0; <http://phytozome.jgi.doe.gov/>) to identify the genome location of the putative *COK-4* paralogs. After the regions with similarity to *COK-4* were identified, they were individually aligned with *COK-4*_SEL1308 (BLASTN pair-wise alignment bl2seq; <http://www.ncbi.nlm.nih.gov/>) to generate the diagrams in the table

^c Schematic representation of genes, adapted from the Phytozome genome view, showing 5' and 3' UTR regions (dark greys rectangles), introns (dark grey lines), and the predict coding regions (light grey rectangles). Black rectangles above the each gene diagram represent the *COK-4*_SEL1308 sequence that aligned with each gene

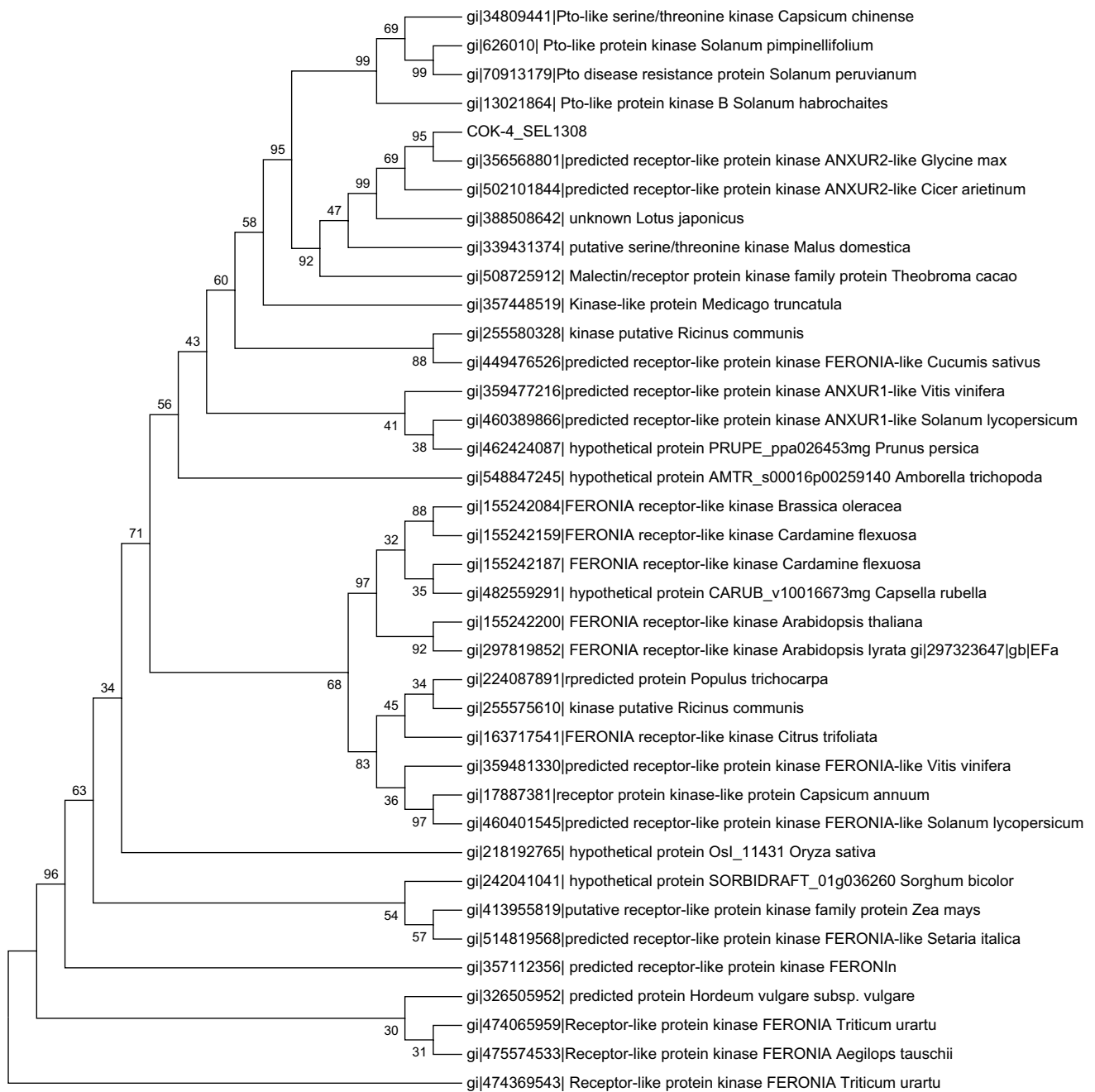


Fig. 3 The COK-4_SEL 1308-predicted protein clustered with members of the *Catharanthus roseus* RLK1-like (CrRLK1L) protein family (mainly FERONIA-like and ANXUR-like) from diverse plant

species. Phylogenetic analysis was performed with the maximum parsimony method using the MEGA 5.05 software (Tamura et al. 2011). Bootstrap support values are adjacent to the tree nodes

located toward the right edge of the *Co-4* locus (Fig. 2b) showed high similarity with the Arabidopsis CrRLK1L family member, FER (BLASTP E-value = 0.0; Table S5). The fourth putative protein with a kinase and malectin domain, encoded by Phvul.008G030200, is similar to a malectin/receptor-like protein kinase from Arabidopsis with no specific function having been established yet (Table S5).

Phylogeny analysis indicates evolutionary relationship between the kinase proteins encoded by genes in the *Co-4* locus and COK-4

A large number of copies of the *COK-4* genes in the region of the *Co-4* resistance locus indicates that gene duplication events may have taken place in this region during the course of the common bean evolution, resulting in the genetic and

phenotypic variations observed among bean lines such as TO and G2333 (Long et al. 2013). Thus, the phylogenetic relationship of proteins similar to COK-4 using the common bean proteome was investigated.

Owing to its highly conserved kinase domain, the COK-4_SEL1308 protein showed significant similarity to protein kinases throughout the common bean genome, including the kinases at the *Co-4* locus. Therefore, these first 100 best hits identified by BLASTP (E-value $\leq 2 \times 10^{-37}$) were used to identify the ones that formed a single clade with COK-4_SEL1308. All of these proteins contain a kinase domain annotated as belonging to the protein superfamily PTKc cd14066 conserved kinase domain (NCBI-conserved domain database), which was considered to perform the phylogeny analysis (Fig. S1). One of the predicted kinases on *Co-4* locus (Phvul.008G029800) showed low similarity to the SEL 1308 COK-4 protein, and it was not in the best 100 kinase matches used for the phylogeny analysis. Also, another kinase at the *Co-4* locus formed a cluster close to the putative COK-4 paralogs (Fig. S1); however, its encoding gene (Phvul.008G031100.1) was not considered a COK-4 copy as it does not have significant nucleotide similarity to the SEL 1308 COK-4 gene. Interestingly, all 18 COK-4 copies located on the *Co-4* locus (Table 2; Table S5) formed a single cluster with COK-4_SEL 1308 (Fig. 4). Four proteins shown to be the closest related to COK-4 form SEL 1308, forming a small sub-clade, which included Phvul.008G028300, Phvul.008G028400, Phvul.008G028500, and Phvul.008G028600 (Fig. 4), confirming the BLASTP results. The four kinases predicted to encode a malectin-kinase protein (Table S5) also formed a single cluster with other RLK proteins from Pv04 (Fig. S1). These results indicate that the kinases present at the *Co-4* locus are closer related to each other than they are to other kinases in the bean genome.

Genes in the *Co-4* locus are transcriptionally regulated during common bean innate immune response

Phylogeny and BLAST analyses indicate that the majority of putative proteins in the *Co-4* locus are similar to CrRLK1L proteins as described above. Members of the CrRLK1L family, such as FER, ANXUR, HERCULES, and THESEUS are known to be involved in plant growth and reproduction (Lindner et al. 2012), but recent results have shown that FER, in particular, is involved also in PAMP-triggered immunity (Keinath et al. 2010). Thus, we reasoned that the predicted kinases at the *Co-4* locus could be regulated by pathogens other than *C. lindemuthianum* as originally identified, and play a role in broad immune response. To test this hypothesis, we used the flg22 peptide found at the N-terminus of bacterial flagellin, which is typically used to assess the PTI response in plants, such as

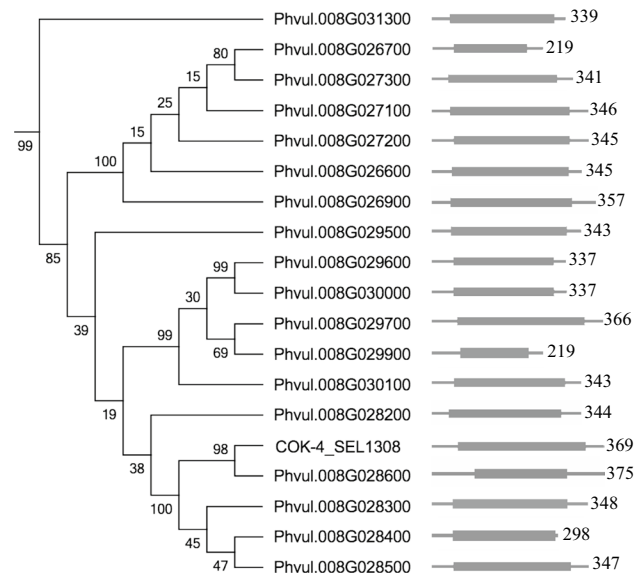


Fig. 4 The COK-4_SEL 1308-predicted protein clustered with common bean kinases located in the *Co-4* genomic region of Pv08. Phylogenetic analysis of predicted amino acid sequence was performed with the maximum parsimony method using the MEGA 5.05 software (Tamura et al. 2011). Bootstrap support values are provided adjacent to nodes. The diagram shown in front of the transcript name represents the single kinase domain (gray rectangles) within the protein. The numbers indicate the total amino acids of each protein. Diagram was adapted from the protein domain view of Phytozome. Only the clade containing the COK-4 is shown (refer to Fig. S1 for the entire tree with the top 100 kinases most similar to COK-4_SEL1308 in the G19833 reference genome)

Arabidopsis, *Lotus japonicus*, and common bean (Navarro et al. 2004; Hou et al. 2011; Lopez-Gomez et al. 2011).

First, we determined whether flg22 could induce PTI in G2333 by assessing callose deposition in treated leaves, a hallmark PTI response in plants (Boller and Felix 2009; Hou et al. 2011). In fact, G2333 leaves showed high numbers of callose deposits 12-h post infiltration. The number of callose deposits decreased after 24 h after flg22 treatment; nonetheless, it was still higher than that of the water control (Fig. 5a and b). To further confirm that flg22 can trigger defense responses in G2333, the expressions of two putative *Arabidopsis* FLS2 orthologs in beans were assessed. The *FLS2-like* (LRR) (Phvul.005G149200) is predicted to have only the leucine-rich repeat (LRR) domain, while the *FLS2-like* (RLK) (Phvul.002G196200) has both LRR and kinase domains similar to FLS2 (Zipfel et al. 2004). FLS2-like (RLK) is the protein with the highest similarity (BLASTP E-value = 0.0 and 44 % identity) to the *Arabidopsis* FLS2 in the bean reference genome. In agreement with the induction of PTI assessed by callose deposition, both *FLS2-like* (LRR) and *FLS2-like* (RLK) were significantly induced by flg22. The upregulation of the *FLS2-like* (RLK) gene lasted until 24 h after flg22 treatment (Fig. 5c).

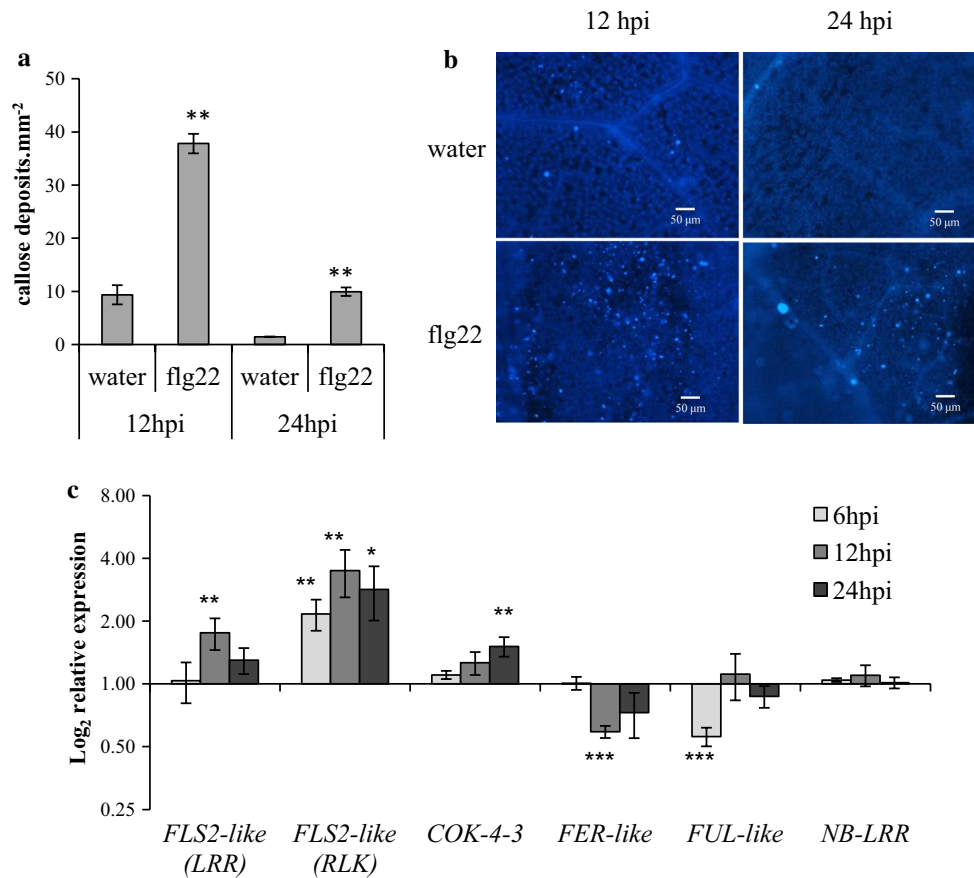


Fig. 5 G2333 responses to the PAMP flg22. **a** Graph shows the average number of callose deposits per mm⁻² of G2333 leaf tissue infiltrated with 1 μ M flg22 or water. Results are shown as average of 108–135 images in three independent biological replicates \pm standard error. **b** Representative images ($\times 100$ magnification) of aniline blue-stained G2333 leaves at 12 or 24 h post-incubation (hpi) with flg22 or water. **c** Expression of the indicated genes (x-axis) in

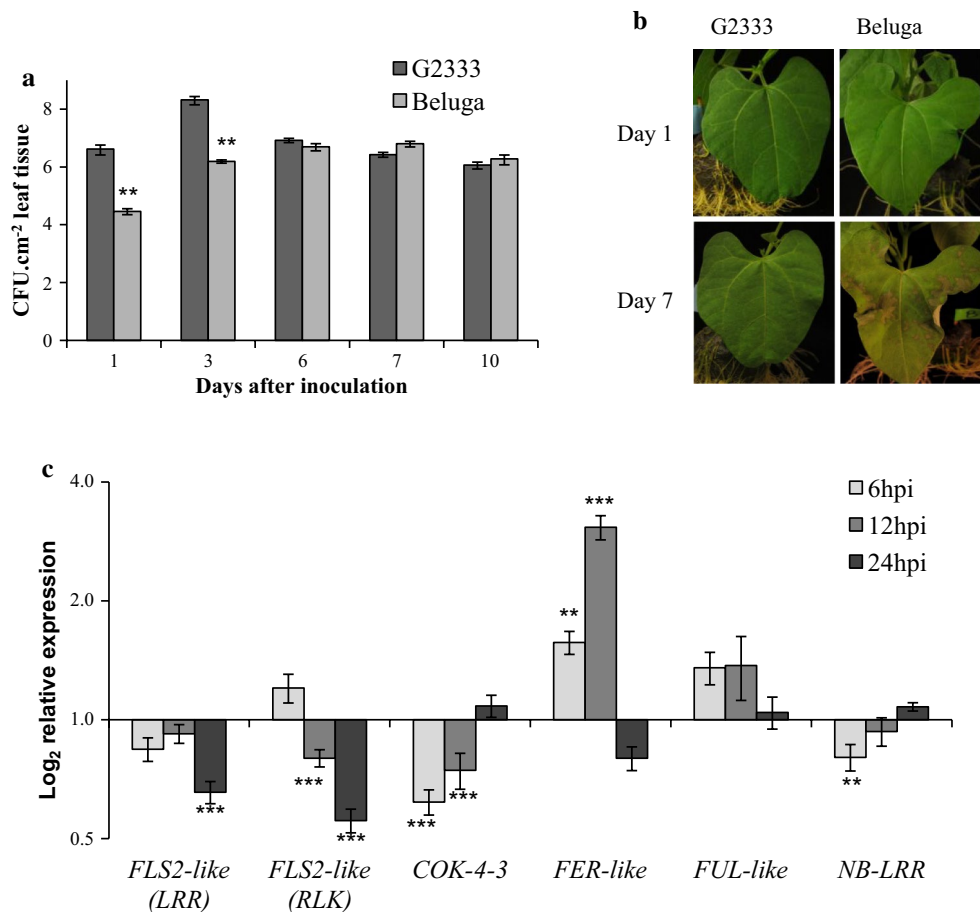
G2333 leaves immersed in 5 μ M flg22 at 6, 12, and 24 hpi relative to their expression in water-immersed leaves (control) considered as 1. Data points are average values of at least two biological replicates ($n \geq 6 \pm$ standard error). Asterisks above the bars of all graphs indicate statistical significance calculated with Student's *t* test (** $p < 0.01$, *** $p < 0.001$)

Next, primers were designed for all genes in the *Co-4* locus, including all *COK-4* copies; however, gene-specific and/or efficient primers could not be obtained for all of them. Thus, we were able to select four genes, which represented different functions in the locus and for which gene-specific and efficient RT-qPCR primers could be designed, to test their expressions after flg22 treatment. Three of them were found to be modulated by flg22: *COK-4-3* (Phvul.008G026900) was significantly induced at 24 hpi, while *FER-like* (Phvul.008G030800) and the putative transcription factor *FUL-like* (Phvul.008G027800) showed transient repressions at early time points and returned to basal levels at 24 hpi (Fig. 5c). Finally, the only NB-LRR domain-encoding gene found at the *Co-4* locus (Phvul.008G031200) was not responsive to flg22 treatment (Fig. 5c).

To determine whether live bacteria also regulate the expression of these genes, G2333 plants were inoculated

with the bacterium *P. syringae* pv. *phaseolicola* (Pph). The G2333 seems to be tolerant to Pph, as these plants supported a large bacterial population in their leaf apoplast since the first day after inoculation (Fig. 6a), and yet, no symptoms were observed even after 7 days post inoculation (Fig. 6b). By contrast, the susceptible cultivar Beluga supported high bacterial titers in the apoplast and showed typical halo blight symptoms later in the infection cycle (Fig. 6a, b). Analysis of gene expression in the inoculated G2333 plants revealed repressions of both *FLS2-like (LRR)* and *FLS2-like (RLK)* genes as bacterial infection progressed (i.e., 12 and 24 hpi), suggesting a low level of defense response that correlated well with high bacterial titer in the leaves. Similarly, SEL 1308 incompatible response to *C. lindemuthianum* involved the repression of PTI pathway and downregulation of the *FLS2-like (LRR)* after fungus infection (Oblessuc et al. 2012). The *COK-4-3* gene was also downregulated as early as 6 hpi, returning to

Fig. 6 G2333 responses to *Pseudomonas syringae* pv. *phaseolicola* (Pph). **a** G2333 showed tolerance to Pph (NPS3121), with no bacterial growth in the leaf apoplast of fully expanded primary leaves dip inoculated with 1×10^8 CFU/ml. **b** Halo blight symptoms were observed 7 days after inoculation only in the Beluga genotype. **c** Expression of the indicated genes (*x*-axis) in G2333 leaves dipped in 10^8 CFU/ml of Pph relative to their expression in mock-inoculated leaves (control) considered as 1. Data points are averages of at least two biological replicates ($n \geq 6 \pm$ standard error). Asterisks above the bars of all graphs indicate statistical significance calculated with Student's *t* test (** $p < 0.01$, *** $p < 0.001$)



normal levels at 24 hpi. In contrast, the *FER-like* gene was upregulated after Pph infection, also returning to normal levels after 24 hpi (Fig. 6c).

The putative transcription factor *FUL-like* showed no change in transcript levels in response to Pph, while the *NB-LRR* domain coding gene was slightly repressed in the initial phase of Pph infection (6 hpi), maintaining normal levels after 12 hpi (Fig. 6c). Altogether, these results suggest that the *Co-4* locus is involved in basal immunity as evidenced by the two kinases that are inversely regulated in plants undergoing immune response (i.e., *flg22* treatment) or infected with a phytopathogen (i.e., large Pph population in the leaves). In addition, our results suggest that other genes in the *Co-4* locus (transcription factors and NB-LRR-resistance gene analogs) might be involved with either resistance or susceptibility as they are only regulated by either *flg22* or Pph infection.

Discussion

Plant responses to pathogens implicate in drastic changes in host genes expressions, and protein turnover resulted from pathogen recognition and activation/inactivation of

a complex chain of metabolic pathways. Overall, the final outcome of the plant response is resistance or susceptibility to the pathogen (Spoel and Dong 2012). Understanding these molecular mechanisms involved in plant immunity is crucial for crop improvement. In the present study, we have determined the most probable location of the *Co-4* locus of common bean, assessed the phylogenetic relationship of the predicted kinases found in the locus, and provided genetic evidence that *Co-4* may have a role in basal immunity in addition to the originally assigned function of its resistance to anthracnose.

The genomic structure of *Co-4* was first analyzed through the molecular mapping of new markers linked to anthracnose resistance. Linkage analysis showed that the newly developed markers PvTA25 and PvSNP_{COK-4}, as well as SAS13 are closely linked to each other and to the *Co-4* resistance gene. The identified genetic distance between the markers, however, may be overestimated due to inherent restrict recombination frequency observed in small mapping populations (Liu 1998), such as the one used here. Thus, these markers could be physically closer to each other than the genetic linkage analysis predicted. Indeed, PvTA25, PvSNP_{COK-4}, and SAS13 markers were located in a small interval also covered by the BAC 78L₁₇

in the common bean chromosome Pv08, confirming that they form a unique locus in the genome. In addition, mismatches between the marker primer sequences and the genome of the bean genotype G19833 indicate that these three markers either could not be amplified by PCR in this bean line or would show different allele size for the PCR amplicon, supporting the linkage of these markers to the resistant allele of the *Co-4* locus.

The G19833 common bean genotype is resistant to some races of *C. lindemuthianum*, such as races 521, 3481, 3977 and 3993, but seems not to contain a resistant allele of the *Co-4* locus (Kelly and Vallejo 2004; Ferreira et al. 2013). Our results revealed that the newly identified markers PvTA25 and PvSNP_{COK-4} amplify the susceptible alleles of the bean line Black Magic showing their transferability across bean genotypes in addition to their being easily assayed with PCR and gel electrophoresis. Thus, these markers may be important tools to be applied in molecular breeding for the development of cultivars containing the resistant allele in the *Co-4* locus.

Genetically linked markers together with the recent release of the common bean genome (Schmutz et al. 2014) enabled us to further refine the genomic structure of the *Co-4* locus. In addition to the markers PvTA25, PvSNP_{COK-4}, SAS13 (Young et al. 1998), all publicly available sequences linked to *Co-4* were located in the common bean genome including the markers SBB14, SH18 (Awale and Kelly 2001), SCARY20 and SCARC08 (Queiroz et al. 2004), as well as the BAC 78L₁₇ and the *COK-4* gene (Melotto and Kelly 2001; Melotto et al. 2004). With the results of this alignment analysis, the most probable region for the locus containing the functional resistance gene was determined to be a 325 Kbp-long sequence at the end of chromosome 8 (Pv08) of the common bean reference genome (G19833 genotype; Schmutz et al. 2014).

Interestingly, this region contains 18 copies of the *COK-4* coding sequence originally identified by Melotto and Kelly (2001), extending the physical boundaries of the *Co-4* locus considerably beyond the BAC 78L₁₇. That BAC, isolated from the bean cultivar Sprite, was reported to have five *COK-4* kinases (Melotto et al. 2004). However, the corresponding region in the G19833 genome contains ten *COK-4* kinases confirming the evolutionary complexity of the locus, where the number of putative *COK-4* paralogs varies according to the bean genotype. Furthermore, all of the *COK-4* coding sequences found on the bean reference genome are transcribed into RNA, as confirmed by RNA-seq and EST mapping analysis (Schmutz et al. 2014), suggesting that they are all active genes. A single *COK-4* related sequence was found in another chromosome (Pv05); however, it may not be functional as no *COK-4* transcript mapped to Pv05. The putative *COK-4* paralogs may collectively contribute to *Co-4*-based resistance, or at

least, one of these genes might be the single functional *Co-4*-resistance gene. These alternatives remain to be experimentally validated.

Previously, *COK-4* was regarded as a *Pto-like* gene, in which all the *COK-4* homologs studied formed a cluster with the *Pto* protein of tomato (Melotto et al. 2004). Surprisingly, the present analysis showed that the a majority (65.2 %) of putative *COK-4* kinases have the highest similarity to the RLK FERONIA (FER, At3g51550) of Arabidopsis, in addition to FER-like proteins from other species, including tomato. Other members of the CrRLK1L kinase subfamily, such as ANXUR, were also found to be similar to *COK-4*. *COK-4* seems to be closely related to *Pto-like* kinases of various *Solanum* species. This apparent discrepancy between previous and current analyses may be due to the much smaller database available at the time of the first study. In addition, *COK-4* may have clustered with *Pto-like* protein from *Solanum* ssp. because these plants are not as well studied as *Solanum lycopersicum* and fully sequenced and/or annotated genomes are still not available. This close phylogenetic relationship among the *COK-4* kinases and only two members of the CrRLK1L family, FER and ANXUR, suggests that the *COK-4*-encoding gene underwent extensive duplication that may or may not have retained the kinase function. Further biochemical analyses are needed to verify the activity of *COK-4* proteins in Pv08.

In plants, genes located in the same region of the genome can be involved in the same pathway and be coregulated, forming operon-like gene clusters (Zmasek and Godzik 2011; Boycheva et al. 2014). The *Co-4* locus might be one such example where *COK-4* paralogs and *FER-like* genes may have evolved from similar functions, and some other genes in this locus, such as the *FUL-like* gene, may be involved in the same pathway. The *FUL-like* (*FRUITFULL*-like) is a putative MADS box transcription factor, and its ortholog in Arabidopsis (AT5G60910) is an *AGAMOUS-like 8* called *FRUITFULL* (*FUL*) because of its involvement in the control of flowering time, fruit development, and determinacy (Pabón-Mora et al. 2012). In addition, the soybean MADS-box transcription factor modulates floral organ numbers, petal identity, and sterility (Huang et al. 2014). Here, the common bean *FUL-like* was co-regulated in the same direction as *FER-like* during PTI induction, suggesting that these genes may have roles in the same response pathway.

In general, CrRLK1L family members seem to be involved, at least in part, in the modulation of reactive oxygen species (ROS) production to regulate cell growth in different developmental stages and hormone signaling pathways, such as ethylene (ET), jasmonate (JA), and salicylic acid (SA), after cell wall damage perception (Wolf et al. 2012). ROS production and activation of ET, JA, and

SA pathways are well-known plant responses to pathogens; thus, CrRLK1L family members are potential membrane receptors that could be active during plant–pathogen interaction. In fact, FER is one member of CrRLK1L family that has been associated with plant immunity so far (Kessler et al. 2010).

The striking similarity between COK-4 and FER prompted us to check whether the *Co-4* locus could be involved in the bean innate immunity. In conditions where bean immunity was activated, i.e., flg22 treatment, we observed that expression of the *COK-4-3* was significantly upregulated along with both *FLS2-like* genes. On the other hand, the *FER-like* gene was strongly repressed during PTI, that is, at the same time points of induction of *FLS2-like* and high callose deposition after flg22 treatment. These data suggest that both *COK-4-3* and *FER-like* genes play distinct roles in PTI responses. While *COK-4-3* may be a positive regulator of PTI, the *FER-like* gene on the *Co-4* locus may be involved in repression of PTI. To further test this hypothesis, the expression of these genes was assessed in bean plants that are tolerant to the bean pathogen Pph (i.e., these plants are symptomless but support high bacterial population in their leaves typical of susceptible interactions). At the same time that the bacterial population was high and *FLS2-like* genes were repressed in these plants indicating low level of PTI, *COK-4-3* was also repressed, and *FER-like* was upregulated. Taken together, these findings provide strong evidence that both *COK-4* and *FER-like* may be involved in the basal immune response to different pathogens.

COK-4 and *FER* may have a common evolutionary origin, but they might have assumed different functions by either *COK-4* losing the malectin domain or *FER* gaining that domain. In cases where evolutionary events in eukaryotes that distinguish a protein from its closest ancestor have been studied, it has been found that domain loss is more common than domain gain and the exchange of a domain is rare (Björklund et al. 2005; Zmasek and Godzik 2011). Thereby, *COK-4* may be a PTI defense-response activator, while *FER-like* may act as PTI inhibitor. Additional studies on the evolution of new biochemical functions emerging in the *Co-4* locus through the *FER-like* and *COK-4* genes should further the current understanding of the molecular pathways underlying bean immunity against a broad range of pathogens. Nonetheless, our results advanced knowledge toward achieving that goal by establishing the boundaries of the *Co-4* locus, providing additional markers for molecular breeding as new tools for employing anthracnose resistance in beans, and reinforcing the role of the putative COK-4 kinases in common bean basal immunity.

Author contribution statement Performed experiments: CF, PRO, MM. Analyzed data: PRO, MM. Conceived and

coordinated the project: MM. Wrote the manuscript: PRO, MM. All authors have read and approved the final version of the manuscript.

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

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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