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Fine mapping of the *Co-4* locus of common bean reveals a resistance gene candidate, *COK-4*, that encodes for a protein kinase

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Abstract The SAS13 SCAR marker, tightly linked with the *Co-4²* gene segregating in a population of 1018 F_2 individual plants, was used as a starting point for cloning gene sequences associated with the *Co-4* locus that conditions resistance to anthracnose caused by the fungal pathogen *Colletotrichum lindemuthianum* in common bean (*Phaseolus vulgaris*). A contig developed from genomic clones flanking the marker region revealed a 1110-bp open reading frame, named *COK-4*. The predicted COK-4 protein contains a serine-threonine kinase domain highly similar to the protein encoded by the *Pto* gene in tomato, but with a highly hydrophobic membrane-spanning region. *COK-4* homologs were cloned and sequenced from different bean cultivars. Single nucleotide polymorphisms were found between the homologous sequences and were confirmed with three restriction enzymes. Restriction patterns among three bean cultivars known to possess different alleles at the *Co-4* locus, SEL 1308 (*Co-4²*), TO (*Co-4*) and Black Magic (*co-4*), were polymorphic. Absolute co-segregation between *COK-4* restriction patterns and the disease phenotype was observed in 96 F_3 families. More than one copy of the *COK-4* gene homolog exists in the bean genome as demonstrated by Southern analysis. These results suggest that *COK-4* is part of the *Co-4* locus conditioning resistance to *C. lindemuthianum* in bean.

Keywords Molecular markers · Serine threonine kinase · Single nucleotide polymorphism · Homologous sequences · Disease resistance gene

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Introduction

Genetic resistance is the most-efficient way to control anthracnose, the disease caused by the fungus *Colletotrichum lindemuthianum* in common bean (*Phaseolus vulgaris* L.). The high genetic variability observed in the pathogen population (Balardin et al. 1997) is associated with different resistance genes present in the host (Balardin and Kelly 1998). One recessive and eight independent dominant resistance genes (*Co-1* to *Co-7, co-8* and *Co-9*) controlling anthracnose in bean have been described (Alzate-Marin et al. 1997; Balardin et al. 1997; Geffroy et al. 1999; Melotto et al. 2000). Each of these genes confers resistance to certain races of the pathogen, strongly suggesting that resistance to anthracnose in common bean follows the gene-for-gene theory (Flor 1947). Certain resistance genes, however, are more effective than others in controlling multiple races of the pathogen (Balardin and Kelly 1998).

The bean breeding line SEL 1308, derived from the highly resistant differential cultivar G2333, is known to possess the single dominant *Co-4²* gene for anthracnose resistance (Young et al. 1998). When inoculated with 34 selected races of *C. lindemuthianum* chosen to represent a diverse sample of the pathogen population, SEL 1308 demonstrated a resistance index (RI) of 97% (Balardin and Kelly 1998). The only cultivar with a higher RI (100%) was G2333 known to possess the combination of three independent resistance genes, *Co-4²*, *Co-5* and *Co-7* (Young et al. 1998). This three-gene combination confers resistance to all described races of the pathogen (Pastor-Corrales et al. 1994). Among the reported resistance genes, the *Co-4²* gene in SEL 1308 exhibits the broadest-based resistance in common bean (Young and Kelly 1996; Balardin and Kelly 1998).

The *Co-4²* gene is a valuable candidate gene for molecular cloning due to its broad resistance and the availability of a tightly linked marker (Young et al. 1998). To better understand the mechanisms of resistance, several disease-resistance genes have been cloned from different plant species. Sequence analysis indicated that these

genes encode structurally similar proteins with conserved domains across plant species (Bent 1996). The opportunity exists, therefore, to identify resistance gene candidates from diverse plant taxa. In common bean and soybean, resistance gene analogs (RGAs) which mapped close to known disease resistance loci were identified using primers specific to conserved regions of known resistance genes from other plant species (Kanazin et al. 1996; Yu et al. 1996; Geffroy et al. 1998, 1999, 2000; Rivkin et al. 1999). Mapping of RGAs has been the main approach used to isolate known resistance gene sequences from common bean. RGAs, however, are not always closely associated with a resistance phenotype and may be loosely linked to a known resistance locus, limiting their value in chromosome walking to the gene. Other approaches, such as map-based cloning, have been used to clone a putative candidate for the *Co-2* gene (Creusot et al. 1999) and are still needed to fine-map and isolate other resistance-gene candidates in common bean.

In the present study the fine mapping of the *Co-4*^{2} locus, using a tightly linked molecular marker, is described. A previously described SCAR marker, SAS13 (Young et al. 1998), was used as the starting point for the cloning of gene sequences associated with the *Co-4*^{2} anthracnose resistance gene. Cloned sequences were compared to amino-acid sequences of known resistance genes in other crops and to nucleotide sequences in bean genotypes with contrasting alleles at the *Co-4* locus.

Materials and methods

Genetic analysis of the segregating population

The bean breeding line SEL 1308 obtained from the International Center for Tropical Agriculture (CIAT), Cali, Colombia, was used as the source of the *Co-4*^{2} gene. This line was derived from a backcross between the anthracnose-susceptible cultivar Talamanca from Costa Rica and the resistant donor parent Colorado de Teopisca (CIAT accession number G2333; Pastor-Corrales et al. 1994) from Mexico. *Co-4*^{2} was not introgressed from a different gene pool or wild relative of *P. vulgaris* and, therefore, recombination events would not be suppressed. SEL 1308 was crossed to Black Magic, a susceptible black bean cultivar (Kelly et al. 1987). Hybrid seeds were advanced to the F_2 generation and a population of 1018 F_2 individuals was developed. Progeny tests were performed in 96 F_2-derived F_3 families to discriminate homozygous from heterozygous resistant genotypes. A total of 1350 F_3 plants were inoculated. Race 73 (ATCC 96512) of *C. lindemuthianum* was chosen to confirm the dominant inheritance of the *Co-4*^{2} gene in SEL 1308. Black Magic, the susceptible parent of the mapping population, dies in 5 days after inoculation. Inoculum preparation, inoculation methods, and disease characterization of the segregat-

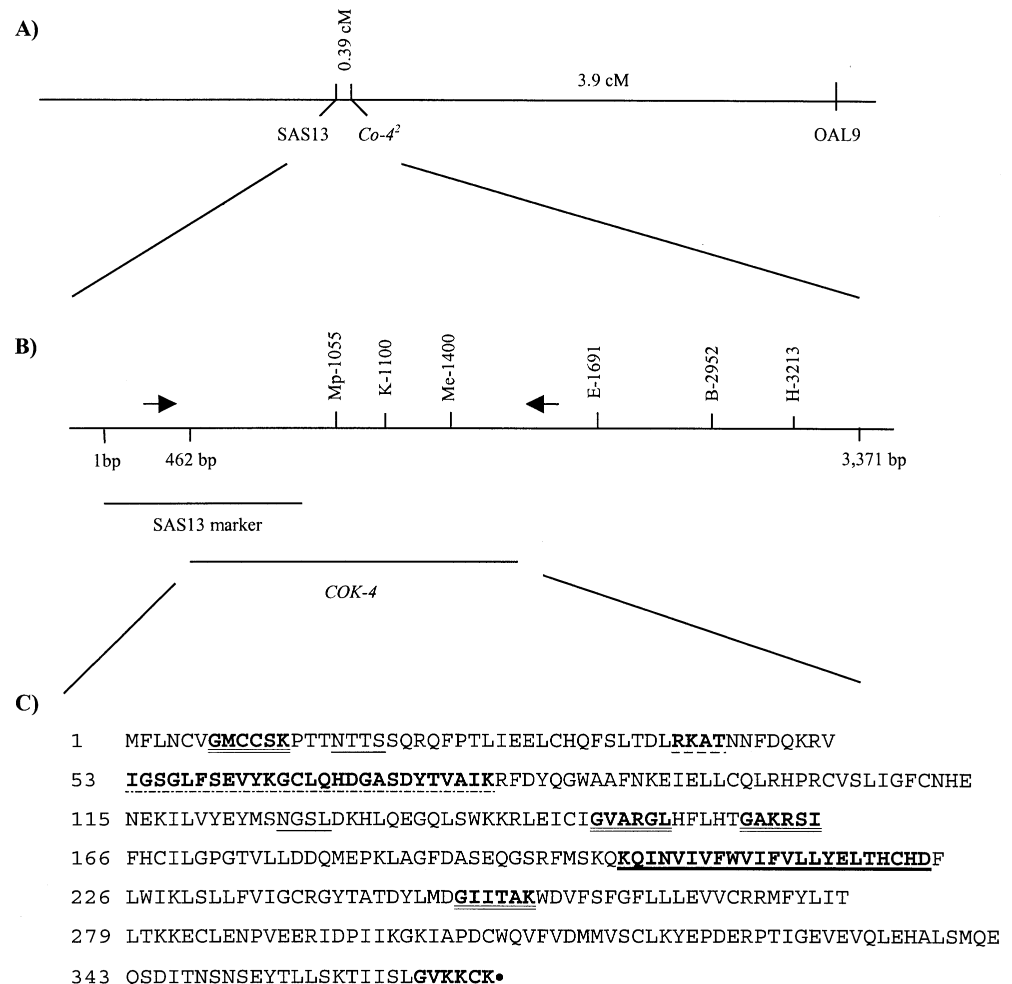

Fig. 1A–C Diagram of the genomic region containing the *Co-4*^{2} locus. **A** Linkage map showing position of molecular markers. **B** Contig developed from overlapping genomic clones showing the position of the SAS13 marker and the *COK-4* gene. *Arrows* indicate *COK-4* specific primers and restriction sites are letter coded, B=*Bam*HI, E=*Eco*RI, H=*Hin*dIII, K=*Kpn*I, Me=*Mse*I, Mp=*Msp*I. **C** Detail of the COK-4 amino-acid sequence showing putative domains: *double underlined*=N-myristoylation sites, *thin underlined*=N-glycosylation sites, *dash underlined*=cAMP and cGMP-dependent protein kinase phosphorylation site, *dot dash underlined*=protein kinase ATP-binding region signature, *thick underlined*=primary transmembrane region, ●=stop codon. The original SAS13 marker site included COK-4 amino acids 1 through 173

ing population were conducted as described by Young and Kelly (1996). Individual F_2 plants from the Black Magic/SEL 1308 population were screened with the SCAR marker SAS13, previously found to be linked to the *Co-4*[2] gene. Procedures for SCAR analysis are described elsewhere (Melotto et al. 1996). Inheritance of both the disease phenotype and molecular markers was confirmed in 1018 F_2 plants using the chi-square test. Linkage analysis was performed using the Linkage-1 software (Suiter et al. 1983) and the distance between the marker and the resistance gene, expressed in centiMorgan (cM), was calculated using Kosambi's function in the Linkage-1 program.

Southern analysis

Genomic DNA from four bean cultivars and a Bacterial Artificial Chromosome (BAC) clone 78L17, kindly provided by Dr. S. MacKenzie, were digested with *Eco*RI according to the manufacturer (Boehringer Mannheim Corporation, Indianapolis, Ind.). The BAC clone from the bean cultivar Sprite (*co-4*[2]/*co-4*[2]) was selected from among four clones that strongly hybridized with our SAS13 probe (Table 1 reported by Vanhouten and MacKenzie 1999). Electrophoresis and blotting were conducted using standard techniques (Sambrook et al. 1989). The *COK-4* ORF was labeled using the DIG High Prime Starter Kit II (Boehringer Mannheim) and used as a probe for hybridization. Stringency washes were performed in 2× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS solutions. Both washes were conducted twice for 30 min at 60°C.

Long-distance PCR and primer walking

DNA clones flanking the SAS13 marker were generated using the Universal GenomeWalker kit (Clontech Laboratories, Inc., Palo Alto, Calif.). DNA from SEL 1308 was purified using phenol and chloroform, and digested with five restriction enzymes, *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I. Adaptors were ligated to restricted DNA samples for PCR-amplification with adaptor-specific primers. PCR reactions were carried out in a 50-μl solution containing 1× Advantage Genomic Polymerase Mix (Clontech Laboratories, Inc., Palo Alto, Calif.), 1.1 mM $Mg(OAc)_2$, 10 mM of each dNTP, 10 pM of each adaptor-specific and SAS13 primers, and 50 ng of DNA template. PCR reactions were placed in a 9600 Thermocycler (Perkin Elmer Applied Biosystems) and the PCR file consisted of seven cycles of 2 s at 94°C, 4 min at 70°C, followed by 32 cycles of 2 s at 94°C, 4 min at 65°C and an extension cycle of 7 min at 65°C. Long-distance PCR (LD-PCR) amplification products were cloned using the TOP TA Cloning kit (Invitrogen Corp., San Diego, Calif.). Both strands of cloned DNA fragments were sequenced using an Applied Biosystems 377 DNA Sequencer (Perkin Elmer Applied Biosystems) as previously described by Melotto et al. (1996). New primers were designed based on those

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a)   1  MFLNCVGMCCSKPTTNTTSSQRQFPTLIEELCHQFSLTDLRKATNNFDQKRVIGSGLFSEVYKGCLQHDG
b)   9                 TNSINDALSSSYLVPFESYRVPLVDLEEATNNFDHKFLIGHGVFGKVYKGVLR-DG
c)   1      MGSKYSKATNSISDASNSFES------YRFPLEDLEEATNNFDKFFIGEGAFGKVYKGVLR-DG
d)  27                                 YRVPFVDLEEATNNFDKFFIGEGGFGKVYRGVLR-DG
e) 564    NVNGGAAASETYSQASSGPRDIHVVETGNMVISIQVLRNVTNNFSDENVLGRGGFGTVYKGEL-HDG
f) 513                                          ATNNFSSANKLGRGGFGTVYKGRLL-DG

a) ASDYTVAIKRFDY-----QGWAAFNKEIELLCQLRHPRCVSLIGFCNHENEKILVYEYMSNGSLDKHL----QEG
b) AKVALKRRTPESS-----QGIEEFETEIETLSFCRHPHLVSLIGFCDERNEMILIYKYMENGNLKRHL----YGS
c) TKVALKRQNRDSR-----QGIEEFGTEIGILSRRSHPHLVSLIGYCDERNEMVLIYDYMENGNLKSHL----TGS
d) TKVALKKHKRESS-----QGIEEFETEIEILSFCSHPHLVSLIGFCDERNEMILIYDYMENGNLKSHL----YGS
e) TK---IAVKRMEAGVMGNKGLNEFKSEIAVLTKVRHRNLVSLLGYCLDGNERILVYEYMPQGTLSQHL----FEW
f) KEIAVKRLSKMSL-----QGTDEFKNEVKLIARLQHINLVRLIGCCIDKGEKMLIYEYLENLSLDSHIFDITRRS

a) Q------L-----SWKKRLEICIGVARGLHFLHTGAKRSIFHCILGPGTVLLDDQMEPKLAGFD---ASEQGSRF
b) D------LPTMSMSWEQRLEICIGAARGLHYLHT---RAIIHRDVKSINILLDENFVPKITDFG---ISKKGT--
c) D------LPSM--SWEQRLEICIGAARGLHYLHT---NGVMHRDVKSSNILLDENFVPKITDFG---LSKTRPQ-
d) D------LPTMSMSWEQRLEICIGAARGLHYLHT---NGVIHRDVKCTNILLDENFVPKITDFG---ISKTMPEL
e) KEHNLRPL-----EWKKRLSIALDVARGVEYLHSLAQQTFIHRDLKPSNILLGDDMKAKVADFGLVRLAPADGKC
f) N------L-----NWQMRFDITNGIARGLVYLHRDSRFMIIHRDLKASNVLLDKNMTPKISDFG---MARIFGRD

a) MSKQKQINVIVFWVIFVLLYELTHCHDFLWIKLSLLFVIGCRGYTATDYLMDGIITAKWDVFSFGFLLLEVVCR
b) -------------------ELDQTH------LSTV-VKGTLGYIDPEYFIKGRLTEKSDVYSFGVVLFEVLCA
c) ------------------LYQTTD-------------VKGTFGYIDPEYFIKGRLTEKSDVYSFGVVLFEVLCA
d) -------------------DLTH--------LSTV-VRGNIGYIAPEYALWGQLTEKSDVYSFGVVLFEVLCA
e) VSVETRL------------------------------AGTFGYLAPEYAVTGRVTTKADVFSFGVILMELITG
f) DAEANTRK-----------------------------VVGTYGYMSPEYAMDGIFSMKSDVFSFGVLLLEIISG

a) R-M----------FY-------LIT-LTKK---------E---CLEN-PVEERIDPII--K-GKIAPDCWQVF--
b) R-S----------AI-------VQS-LPREMVNLAEWAVE---SHNNGQLEQIVDPNL--A-DKIRPESLRKF--
c) R-S----------AM-------VQS-LPREMVNLAEWAVE---SHNNGQLEQIVDPNL--A-DKIRPESLRKF--
d) RPA----------LY-------LSE-MMSS---------DDETQKMG-QLEQIVDPAI--A-AKIRPESLRMF--
e) R-KALDETQPEDSMH-------LVTWFRRM---------Q---LSKD-TFQKAIDPTI--DLTEETLASVSTV--
f) K-KNNG-------FYNSNQDLNLLA-LVWR---------K---WKEG-KWLEILDPIIIDS-SSSTGQAHEILRC

a) VDMMVSCLKYEPDERPTIGEVEVQLEHALSMQEQSDITNSNSEYTLLSKTIISLGVKKCK   369
b) GDTAVKCLALSSEDRPSMGDVLWKLEYALRLQE                              318
c) GETAVKCLALSSEDRPSMGDVLWKLEYALRLQE                              308
d) GETAMKCLAPSSKNRPSMGDVLWKLEYALCLQE                              312
e) AELAGHCCAREPHQRPDMGHAVNVLSTLSDVWKPSDPDSDDS                     902
f) IQIGLLCVQERAEDRPVMASVMVMI                                      792
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Fig. 2a–f Alignment from regions of similarity between the COK-4 protein sequence and reported protein sequences. Amino-acid identity is indicated in *bold-thick underlined* letters and amino-acid similarity is indicated in *thin underlined* letters. *Numbers* in the sequence indicate the first and last amino acids aligned. **a** COK-4 protein (GenBank data base accession no. AF153441); **b** disease resistance protein kinase Pto gi|430992; **c** serine/threonine protein kinase Pto (*L. esculentum*) gi|1809257; **d** putative serine/threonine protein kinase, *Fen* gene (*L. esculentum*) gi|557882; **e** TMK (*O. sativa*) gnl|PID|e267533; **f** extracellular S domain of *B. oleracea* gnl|PID|e1172841

sequences to walk in uncloned genomic DNA as proposed by Siebert et al. (1995).

Sequence analysis

The sequences obtained by primer walking were aligned and a contig was generated using the Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, Mich.). The consensus sequence was compared to other sequences available in the computer database using BLAST search programs (Altschul et al. 1997). The amino-acid sequence deduced from the consensus DNA sequence was analyzed for putative function and domains, using the computer search programs PROSITE (Hofmann et al. 1999), SOSUI (Hirokawa et al. 1998) and BLASTP (Altschul et al. 1990).

Restriction analysis of specific PCR products

PCR primers were designed to amplify specific DNA fragments near the SAS13 marker region. The PCR amplification reaction contained 50 ng of genomic DNA, 10 mM of each dNTP, 10 pmol of each forward and reverse primer, 1× enzyme buffer containing $MgSO_4$ and 1 U of *Pfu* DNA polymerase (Promega, Madison, Wis.). PCR reactions were placed in a 9600 Thermocycler (Perkin Elmer Applied Biosystems) and the PCR file consisted of 34 cycles of 20 s at 95°C, 30 s at 55´°C, and 4 min at 72°C, followed by an extension cycle of 7 min at 72°C. The amplification product was used in a digestion reaction containing 1× enzyme buffer, 10 U of restriction enzyme and 10 µl of PCR reaction. Digestion of the DNA fragment was carried out for 4 h at 37°C. Restriction patterns were observed an on 0.8% ethidium bromide-stained agarose gel.

Results

The SCAR marker SAS13 was previously found to be tightly linked to the *Co-4*[2] gene, which conditions resistance to anthracnose in common bean (Young et al. 1998). The marker co-segregated with 1014 F_2 individuals in a population size of 1018 (Fig. 1A). Four recombinant individuals were found and the genetic distance between SAS13 and *Co-4*[2] was estimated at 0.39 cM. The 950-bp DNA fragment generated by the SAS13 marker was sequenced and analyzed for similarities to the sequences of known disease resistance genes. The alignment obtained by using BLAST search software (Altschul et al. 1997) revealed a high similarity to serine-threonine kinase (STK) domains, such as the ones encoded by the disease resistance gene *Pto* (gi|430992; gi|1809257; 38% identity, 53% similarity and 15% gap; Martin et al. 1993) and the *Fen* gene (gi|1098334; 37%, 51%, 12%; Martin et al. 1994) in tomato. Other proteins similar to the SAS13 DNA fragment included receptor-like kinases (RLK) from other organisms including *Arabidopsis thaliana*, *Brassica* sp., *Oryza sativa* and *Zea mays*. Based on these results, the SAS13 marker was used as a starting point for primer walking in genomic DNA to find complete gene sequences encoding for protein kinase domains. Four overlapping clones extending the original SAS13 950-bp fragment were obtained and the full length of the contig included 3371 bp (Fig. 1B). Primer pairs were designed to test whether the generated clones were contiguous in the plant genome. All primer sets amplified a single band of the predicted size (data not shown).

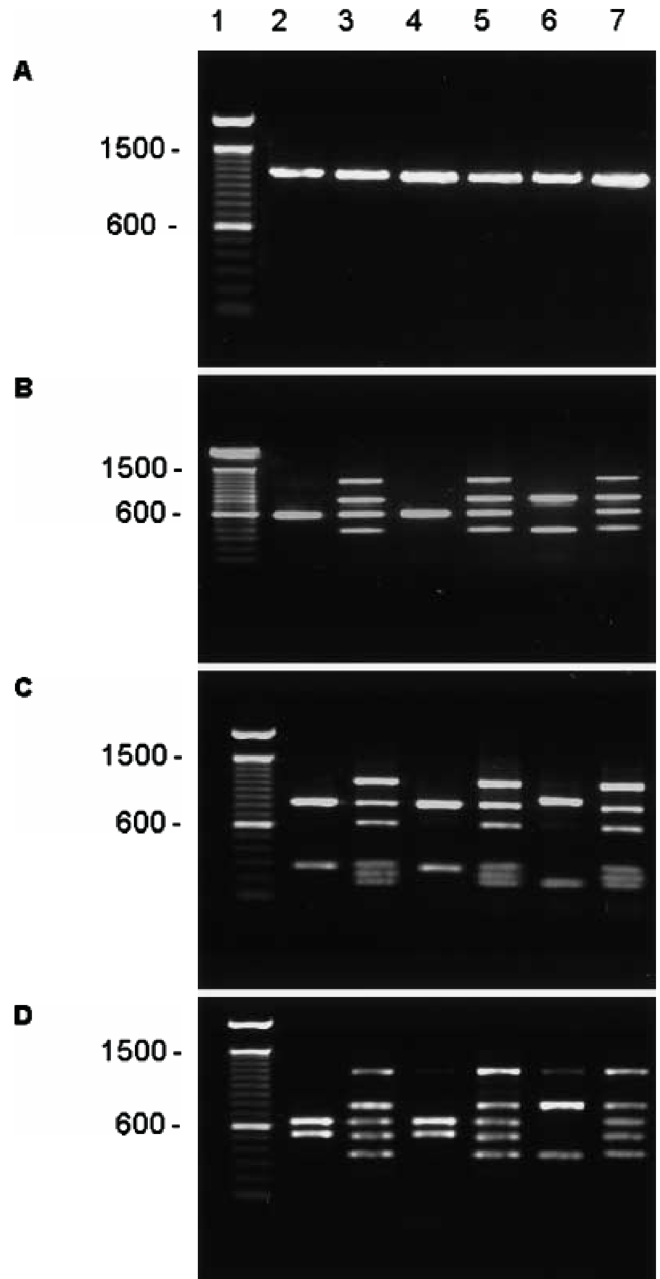

Fig. 3 A–D Restriction analysis of the *COK-4* amplified in several genotypes. **A** Undigested; **B** *Kpn*I; **C** *Mse*I; **D** *Msp*I. *Lane* (*1*) 100-bp DNA ladder, (*2*) SEL 1308 (*Co-4*[2]/*Co-4*[2]), (*3*) heterozygous resistant F_2 plant (*Co-4*[2]/*co-4*[2]), (**4**) homozygous resistant F_2 plant (*Co-4*[2]/*Co-4*[2]), (*5*) homozygous susceptible F_2 plant (*co-4*[2]/*co-4*[2]), (**6**) TO (*Co-4*/*Co-4*), (*7*) Black Magic (*co-4*[2]/*co-4*[2])

Sequence analysis of the contig revealed an open reading frame (ORF) of 1110 bp, which was named *COK-4* (Fig. 1 C). Two essential eukaryote promoter elements, TATA and CAAT boxes, and putative promoter sequences were found upstream of the *COK-4* gene. The predicted amino-acid sequence of *COK-4* has a high degree of similarity with expressed sequences generated

A) 1 atgtttctgaattgtgtgggcatgtgttgttcgaagcccacaacaaatacaacttcatct
B) atgtttctgaattgtgtgggcatgtgttgttcgaagcccacaacaaatacaacttcatct
C) atgtttctgaattgtgtgggcatgtgttgt**a**cgaagcccacaacaaatacaacttcatct

A) 61 cagagacagtttccaacgttgatagaagagctgtgccatcaatttctctcaccgatctt
B) cagagacagtttccaacgttgatagaagagctgtgccatcaatttctctcaccgatctt
C) cagagacagtttccaacgttgatagaagagctgtgccatcaatttctctc**t**ccgatctt

A) 121 aggaaagccaccaataactttgatcagaagagagtaataggaagtggattatttagtgaa
B) aggaaagccaccaataactttgatcagaagagagtaataggaagtggattatttagtgaa
C) aggaaagcca**t**caataactttgatcagaagagagtaataggaagtggatt**t**tttag**g**gaa

A) 181 gtatacaaagggtgtctgcagcacgatggtgcttctgattacacggtcgcaataaagcga
B) gtatacaaagggtgtctgcagca**t**gatggtgcttctgattacacggtcgcaataaagcga
C) gtata**tt**aagggtgtctgcagca**t**gatggtgcttctgattacacggtcgcaataaagcga

A) 241 tttgattatcaaggatgggcagcgttcaacaaggaaatcgaattgctatgccagcttcgt
B) tttgattatcaaggatgggcagcgttcaacaaggaaatcgaattgctatgccagcttcgt
C) tttgattatcaaggatggg**a**agcgttcaacaaggaaatcgaattgctatgccagcttcgt

A) 301 caccctagatgtgtttctcttataggattctgcaaccacgaaaatgagaagattcttgta
B) caccctagatgtgtttctcttataggattc**a**gcaaccacgaaaatgagaagattcttgta
C) caccctagatgtgtttctcttataggattctgcaaccac**c**aaaatgagaagattcttgta

A) 361 tacgagtacatgtccaatggatctctagataaacacctacaagaaggtcaactatcatgg
B) tacgagtacatgtccaatggatctctagataaacacctacaagaaggtcaactatcatgg
C) tacgagtacatgtccaatggatctctagataaacacctacaaga**t**ggt**g**aactatcatgg

A) 421 aagaagaggctggagatatgcataggagtagcacgtggactacacttccttcacaccgga
B) aagaagaggct**a**gagatatgcataggagtagcacgtggactacact**a**ccttcacaccgga
C) aagaagaggct**a**gagat**c**tgcataggagtagcacgtggactacact**a**ccttcacac**t**ggt

A) 481 gccaagcgttccatctttcactgtatcctcggtcctggtaccgtccttttggatgaccag
B) gccaagcgttccatctttcactgtatcctcggtcctggtaccgtccttttggatgaccag
C) gccaagcgttccatctttcactgtatcctcggtcct**a**gtacc**a**tccttttggatgaccaa

A) 541 atggagccaaaactcgctggtttcgatgctagcgagcagggatcacgttttatgtcaaag
B) atggagccaaaactcgctggtttcg**g**tgctagcgagcagggatcacgttttatgtcaaag
C) atggagccaaaactcgctggtttcg**g**tg**t**tagc**at**gcagggatcacgttttatgtcaaag

A) 601 cagaagcaaatcaatgt-gatcgtgttttgggtaatttttgttttgttgtatgagctcac
B) cagaagcaaatcaatgt**a**gatcgtgttttgggtaatttttgtttt**t**ttgtatgagctcac
C) cagaagcaaatcaatgt**a**gatcgtgttttgggtaatttttgttttgttgtatgagctcac

A) 660 tcactgccatgatttttgtggatcaaactaagct--tactctttgttataggttgtagggg
B) tcactgccatgatttttgtggatcaaactaagct--tactctttgttataggttgt**t**gggg
C) tcactgc**a**atga**a**ttttgtggatcaaactaagct**aa**tactctttgttataggt**ac**t**ttt**gg

A) 720 ctacacggctacggactatctcatggatggtatcatcacagctaaatgggatgttttctc
B) ctacacggctacggactatctcatggatggtatcatcacagctaaatgggatgttttctc
C) ctac**c**cggctacggactat**g**tcatggatggta**c**catcacagctaaatgggatgttttctc

A) 780 atttggtttccttctactagaagttgtgtgcaggaggatgttttatttaataactctgac
B) atttggtttccttctactagaagttgtgtgcaggaggatgttttattt**g**ataactctgac
C) atttggtttccttctactagaagttgtgtgcaggaggatgttttattt**g**ataactctgac

A) 840 taaaaaagaatgtctggagaatcctgttgaggagagaattgatccgattatcaaaggaaa
B) taaaaaa**a**aatgtctggagaatcctgttgag**t**agagaattgatccgattatcaaagg**g**aa
C) taaaaaa**a**aatgtctggagaatcctgttgaggagagaattgatccgattatcaaagg**g**aa

A) 900 gattgcaccagattgttggcaagtgtttgtagatatgatggtaagttgcttgaagtatga
B) gattgcaccagattgttggcaagtgtttgtagatatgatggtaa**c**ttgcttgaagtat**a**a
C) gattgcaccagattgttggcaagtgtttgtagatatgatggtaa**c**ttgcttgaagtatga

A) 960 accagatgagagaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat
B) accagatgagagaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat
C) accagatgag**c**gaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat

A) 1020 gcaggaacaatctgatatcacaaactccaactctgagtataccttactctccaaaaccat
B) gcaggaacaa**g**ctgatatcacaaactccaactctgagtatac**t**ttact**g**tccaaaaccat
C) gcaggaacaa**g**ctgatatcacaaactccaactctgagtataccttact**g**tccaaaaccat

A) 1080 tatttcccttggagtgaagaaatgtaagtga 1110
B) tatttccct**g**ggagtgaagaaatgtaagtga
C) tatttccc

◀ **Fig. 4A–C** DNA sequence showing single nucleotide polymorphisms (SNPs) in *underlined bold letters* among three cultivars possessing different *COK-4* homologs, **A** SEL 1308, **B** Black Magic, and **C** TO. SEL 1308 and Black Magic are 98% identical, SEL 1308 and TO are 95% identical, and Black Magic and TO are 94% identical

by the *Pto* gene from *Lycopersicon pimpinellifolium* and *Lycopersicon esculentum* (Martin et al. 1993), the *O. sativa* RLK protein *Xa21* gene (gi|1122442; 30%, 41%, 8%; Song W-Y et al. 1995), the TMK protein from rice (29%, 45%, 16%; van der Knaap et al. 1996), the extracellular S-domain from *Brassica oleracea* (gi|2598269; 34%, 48%, 15%) (Fig. 2), the S-domain receptor-like protein kinase from *Z. mays* (gi|3445397; 33%, 49%, 14%), and the leucine-rich repeat (LRR) transmembrane protein kinase 2 from *Z. mays* (28%, 45%, 19%; Li and Wurtzel 1998). No similarity was observed with the NBS-LRR nucleotide or protein sequences in bean (AF098969; Creusot et al. 1999). The protein encoded by *COK-4* was analyzed for possible functional domains. The COK-4 protein has a STK domain, which includes a protein kinase ATP-binding region signature (amino acids 53 to 79), a primary transmembrane domain (amino acids 202 to 224), putative sites for N-myristoylation and N-glycosylation, and a cAMP- and cGMP-dependent protein kinase site (amino acids 41 to 44) (Fig. 1C).

Two specific primers were designed to amplify the *COK-4* gene. The forward primer sequence is 5′-GTATGGTAAGTGACAAGTGAGA-3′ and the reverse primer sequence is 5′-ACCTGGTCACTTACATTTCT-

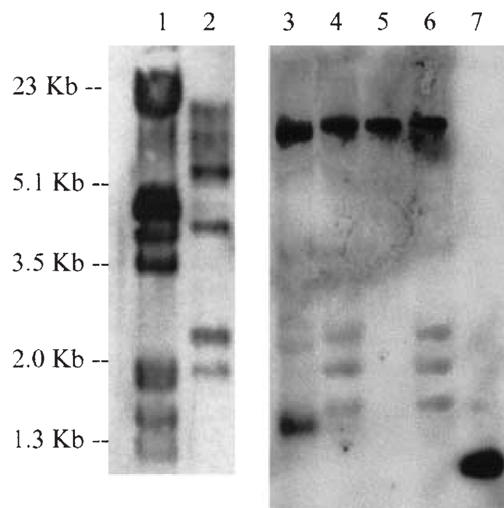

Fig. 5 Southern analysis of *Eco*RI-digested DNA of bean cultivars hybridized with the *COK-4* ORF. *Lane* (*1*) Molecular Marker III (Boheringer Mannheim), (*2*) BAC 78L17, (*3*) SEL 1308, (*4*) Back Magic, (*5*) TO, (*6*) SEL 1360, (*7*) *COK-4* ORF. The molecular weight is indicated in kb

TCA-3′. PCR analysis using those primers confirmed the presence of a single 1150-bp DNA fragment in the bean genome, which contains the *COK-4* gene (Fig. 3A). Both parents of the mapping population and all individual bean cultivars tested possessed the *COK-4* gene. To search for nucleotide polymorphisms between genotypes, the 1150-bp DNA fragments from both the resistant parent SEL 1308 and the susceptible parent Black Magic were cloned and sequenced. Alignment of these two sequences revealed a nucleotide identity of 98% between resistant and susceptible parents (Fig. 4). Single nucleotide polymorphisms (SNP) were observed as nucleotide substitutions, deletions, or insertions. The predicted amino-acid sequence from the susceptible parent was interrupted by several stop codons (data not shown). Based on the sequence data a restriction map at that region was predicted (Fig. 1B) and confirmed with 15 different restriction enzymes in the resistant parent SEL 1308 and susceptible parent Black Magic (data not shown). To further demonstrate the presence of SNPs between the parental genotypes and to confirm the DNA sequence, *COK-4* was digested with specific restriction enzymes. Three restriction enzymes, *Kpn*I, *Mse*I and *Msp*I, were polymorphic between the resistant and susceptible parents of the mapping population (Fig. 3B–D). All three enzymes restricted the *COK-4* of SEL 1308 at one site and the *COK-4* of Black Magic at two or more sites. Restriction of SEL 1308 with *Kpn*I appears as a single band (Fig. 3B) as the restriction products were similar in size (Fig. 1B). Co-segregation of *COK-4* restriction patterns with the disease phenotype was tested in 96 F_3 families originating from the Black Magic/SEL 1308 cross. All individuals genotyped (on 1350 F_3 individual plants) as carrying the resistance allele (homozygous dominant plants) had only one restriction site similar to SEL 1308, whereas all plants carrying the susceptible allele (homozygous recessive and heterozygous) had more than one restriction site similar to Black Magic. No recombinants were found. The four recombinant individuals between SAS13 and *Co-4*^{2} were analyzed for the *COK-4* gene. Three susceptible plants carrying SAS13 possessed the *COK-4* allele of Black Magic, and the heterozygous resistance plant without the SAS13 marker possessed one allele of Black Magic and the other allele of SEL1308, as expected in a heterozygous plant. Restriction analysis of the bean cultivar TO, known to possess a different resistance allele at the *Co-4* locus (Young and Kelly 1996; Young et al. 1998) revealed a third restriction pattern (Fig. 3B–D). The *COK-4* homolog found in TO was sequenced and aligned with the *COK-4* homologs present in SEL 1308 and Black Magic (Fig. 4). The TO and SEL 1308 nucleotide sequences were 95% identical whereas the alignment of the amino-acid sequences revealed 86% identity, 94% similarity, and 1% gap. The nucleotide sequences of TO and Black Magic were 94% identical.

To determine the copy number of the *COK-4* gene in different bean cultivars, *Eco*RI-restricted DNA was probed with the *COK-4* ORF (Fig. 5). The restriction enzyme *Eco*RI does not cut the *COK-4* gene. The resistant cultivar SEL 1308 possessed two major homologous DNA sequences of 1.5 and 9 kb in size, whereas the susceptible cultivars SEL 1360 and Black Magic possessed multiple homologous sequences of various sizes. Again, TO possessed a unique RFLP pattern with only one 9-kb DNA fragment. BAC clone 78L17 that spanned the *Co-4* locus was also restricted with *Eco*RI and hybridized with the *COK-4* ORF. At least three copies of this gene are clustered in the BAC clone (Fig. 5).

Discussion

Two lines of evidence strongly suggest that the *COK-4* gene, herein described, is a member of the *Co-4*^{2} locus conditioning resistance to anthracnose in common bean. First, genetic analysis indicated co-segregation of the *COK-4* gene with the resistant phenotype in a segregating population of 96 F_3 families. Secondly, amino-acid sequence analysis of the *COK-4* gene, which is located 462-bp downstream from the SAS13 forward primer (Fig. 1B), revealed high similarity with previously cloned resistance genes and protein domains known to play an important role in disease resistance. The putative protein encoded by the *COK-4* gene has the structure of STKs and also aligns with RLKs. Receptor-like kinases contain an extracellular domain possibly functioning in ligand binding and a cytoplasmic domain responsible for signal transduction (Walker 1994). The COK-4 protein most likely is localized at the membrane because it contains three highly hydrophobic regions characteristic of a transmembrane domain and has an average hydrophobicity of –0.036 (calculated by the SOSUI software; Hirokawa et al. 1998). Alignment of the *COK-4* amino-

acid sequence with the extracellular S-domain of *B. oleracea* (gi|2598269) and LRR transmembrane and RLK domain of *Z. mays*, also supports localization of the COK-4 protein in the cellular membrane. If the resistance gene product is the receptor for the pathogen Avr gene product, it is expected that recognition occurs at the membrane level. *C. lindemuthianum* is a hemibiotrophic fungus that penetrates the bean cell wall (Bailey et al. 1992). In addition, race specificity in *C. lindemuthianum* is expressed after fungal penetration through the epidermal cell wall, and the primary hyphae of *C. lindemuthianum* remain external to the host plasma membrane, which becomes invaginated around the fungus (Bailey et al. 1992). These observations suggest that the avirulence gene product may be a host-specific elicitor either secreted and/or located in the membrane, and that pathogen recognition may occur at the surfaces of the *C. lindemuthianum* infection hyphae and bean cell membrane.

Many of the previously cloned disease resistance genes that confer resistance to bacterial diseases appear to be localized in the cytoplasm. For instance, Pto is a soluble protein localized in the cytoplasm of tomato cells where it binds to the AvrPto protein of the bacterial pathogen (Scofield et al. 1996; Tang et al. 1996). Bacterial *Avr* gene products are known to be secreted into the host cytoplasm through the type III secretory system (Bent 1996). However, the predicted protein product of the *Xa21* gene that confers race-specific resistance to the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* of rice, carries LRR motifs in the extracellular domain and STK in the cytoplasmic domain (Song et al. 1995). Less is known about the function of Avr proteins from fungal pathogens and only a few fungal Avr-generated signals have been described. One well-studied example of race-cultivar specificity is the *Cladosporium fulvum/Lycopersicon* pathosystem (Joosten and de Wit 1999). Since colonization of tomato by *C. fulvum* is restricted to the apoplast, exchange of molecular signals between the fungus and plant has to occur extracellularly. All *Cf* genes are presumed to encode extra-cytoplasmic membrane-anchored proteins that contain LRRs. The Cf proteins possess domains that can act directly as extracellular receptors for Avr protein elicitors secreted by *C. fulvum* (Jones et al. 1994; Dixon et al. 1996; Joosten and de Wit 1999). Although Avr proteins of *C. lindemuthianum* have not been isolated, the occurrence of race-cultivar specificity suggests the presence of an Avr-generated signal triggering plant defense response. Based on the similarities between the *C. fulvum*/tomato and *C. lindemuthianum*/bean pathosystems, one would expect that host-specific elicitors and anthracnose resistance gene products are located at the membrane where the pathogen is recognized.

Although the *COK-4* region was amplified in both resistant and susceptible parents of the mapping population, internal differences in nucleotide sequences exist as indicated by restriction (Fig. 3) and sequence (Fig. 4) analyses. Single nucleotide polymorphisms (SNPs) identified in the *COK-4* sequences of resistant and susceptible bean lines and co-segregation of restriction patterns with the disease phenotype, strongly suggest that the *COK-4* gene is involved in anthracnose resistance. Small variation in gene sequences can result in contrasting phenotypes. In tomato, the *Pto* and *Fen* genes are present in bacterial speck-susceptible and fenthion-sensitive genotypes and encode a protein kinase 87% and 98% identical to the resistance alleles, respectively (Jia et al. 1997). Two functional *Cf-2* resistance genes in tomato differ in only three amino acids near the C-terminus region (Joosten and de Wit 1999). A SNP, found in rice, accounted for 80% of the variation in amylose content (Ayres et al. 1997). The original SAS13 SCAR marker, which amplified a single 950-bp fragment in the resistant parent, co-segregated with the *Co-4*^{2} resistance gene in the segregating population of 1018 F_2 individuals. Four recombinant individuals between the marker and the *Co-4*^{2} locus were identified. Three susceptible individuals possessing the SAS13 fragment, and one resistant line lacking the fragment, were observed. These four F_2 individuals, however, possessed the *COK-4* allele corresponding to the disease phenotype of the plant confirmed by restriction analysis (data not shown). This result suggests that recombination events may have occurred within the *Co-4* locus that may span a region as large as 0.39 cM. In addition, other gene sequences that code for LRRs at this locus may be required for full expression of resistance. In this study, additional copies of the *COK-4* ORF were identified (Fig. 5). The physiological function of the *COK-4* gene could not be detected because efficient transformation protocols have not been developed in common bean.

Previous genetic studies indicated that the *Co-4* locus is a complex gene family. Two resistance alleles that reside at the *Co-4* locus have been described. One allele present in the bean cultivar TO and the other present in SEL 1308 were supported by allelism tests (Young et al. 1998) and DNA sequence analysis. TO showed a unique restriction pattern with 44 SNPs at the *COK-4* region compared to SEL 1308. Classic genetic analysis indicates a single gene segregating in the Black Magic/SEL 1308 F_2 mapping population; however, other genes may be tightly clustered at the *Co-4*^{2} locus. Based on Southern analysis, bean cultivars appear to possess multiple copies of the *COK-4* gene that are clustered. If the *COK-4* homolog in TO is non-functional and different from that in SEL 1308, clearly the functional *Co-4* gene in TO may be a gene duplication based on the RFLP patterns and must be linked to *COK-4*. Another anthracnose resistance gene, *Co-2*, has also been shown to be a complex multigene family. Sequence analysis of a linked marker revealed multiple copies of LRR sequences clustered in the vicinity of the *Co-2* gene (Geffroy et al. 1998). In addition the isolation of three NBS-LRR sequences including two cDNAs suggests that the *Co-2* locus is highly complex and most likely harbors functional genes of both LRR and the kinase proteins involved in resistance (Creusot et al. 1999). An ancestral gene cluster was also identified in the vicinity of the *Co-9* gene on

bean linkage group B4 (Geffroy et al. 1999). Resistance genes appear to be clustered in the plant genome and may occur in multiple copies spanning large regions of the plant genome (Kesseli et al. 1993; Maisonneuve et al. 1994; Meyers et al. 1998). RAPD markers flanking the *Co-4*^{2} gene (data not shown) are being used to further investigate the presence of a gene cluster at the *Co-4* locus and to assist in the location of the *Co-4*^{2} gene on the integrated bean linkage map (Freyre et al. 1998).

These findings indicate that tightly linked molecular markers may be used to identify disease resistance gene candidates. The SAS13 marker linked to the *Co-4* locus, allowed the identification of different resistance alleles present in diverse bean cultivars. The marker was used to clone the *COK-4* gene from resistant cultivars in addition to homologs present in susceptible cultivars. The *COK-4* gene, that appears to condition resistance to a fungal pathogen of common bean, is highly similar to the *Pto* resistance gene present in tomato. By comparing the *COK-4* homologs in different bean genotypes, SNPs were identified and were more accurate than the tightly linked SCAR marker in discriminating the plant genotype at the *Co-4* locus. Most important, SNPs co-segregated with the disease phenotype in a large (>1000) segregating population and could be used to identify three different alleles at the *Co-4* locus. Until genetic complementation experiments are available in bean, it will not be possible to definitively assign a function to cloned genes. We are limited to indirect evidence such as genetic segregation and sequence comparison to find the best candidate gene. The bean pathogen *C. lindemuthianum* is host-specific, limiting the opportunity to test the function of disease resistance genes in model organisms. This work represents the first report of the successful cloning and molecular characterization of a likely candidate for the disease resistance gene in common bean. Molecular cloning of resistance genes should facilitate studies on plant-pathogen interaction and ultimately facilitate the genetic improvement of crop species.

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