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Maeli Melotto

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James D. Kelly
Major professor

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GENETIC INHERITANCE AND MOLECULAR CLONING OF ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

By

Maeli Melotto

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ABSTRACT

GENETIC INHERITANCE AND MOLECULAR CLONING OF ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

By

Maeli Melotto

Resistance to anthracnose caused by *Colletotrichum lindemuthianum* appears to be controlled by independent complex loci. New sources of resistance have been identified and genetic and molecular characterization indicated that there are fewer loci with multiple allelic series than expected. Two diverse loci conditioning resistance to anthracnose in common bean (*Phaseolus vulgaris* L.) were investigated. Genes conditioning resistance in the Andean bean cultivars Kaboon and Perry Marrow were shown to be allelic to the *Co-1* gene present in Michigan Dark Red Kidney (MDRK). The gene symbols $Co\text{-}1^2$ and $Co\text{-}1^3$ were assigned to these alleles. Other putative alleles were recognized at this complex locus. The second locus, $Co\text{-}4^2$, present in the Middle American differential cultivar G 2333 was characterized at the molecular level. A RAPD marker, $OAS13_{950}$, tightly linked to the $Co\text{-}4^2$ was identified. A SCAR marker (SAS13), developed from the $OAS13_{950}$ RAPD marker, was dominant and polymorphic similar to

the original RAPD, and supported the tight linkage between the marker and the *Co-4*^{2} allele. The markers were present in two other differential cultivars, not previously characterized, and in four navy bean cultivars suggesting the existence of a gene family for anthracnose resistance at or near the *Co-4* locus. Molecular markers, tightly linked to resistance genes, were used to facilitate the identification of an uncharacterized resistance gene for which no discriminating race of the pathogen is currently known. The SAS13 marker was used as a starting point for cloning gene sequences associated with the *Co-4* locus. A contig developed from genomic clones flanking the marker region revealed an 1110 bp open reading frame, named *COK-4*. The predicted COK-4 protein contains a serine-threonine kinase domain with a highly hydrophobic membrane-spanning region. Specific primers designed to amplify the *COK-4* region, were used to clone and sequence *COK-4* homologs from different bean cultivars. Single nucleotide polymorphisms (SNP) found between the homologous sequences were further confirmed with three restriction enzymes. Restriction patterns were polymorphic among three bean cultivars known to possess different alleles at the *Co-4* locus confirming the presence of different alleles at anthracnose resistance loci. These results strongly suggest that the *COK-4* gene is a member of the complex *Co-4* locus conditioning resistance to anthracnose in bean. *COK-4*, a gene ortholog of the *Pto* in tomato, is the first disease resistance gene successfully cloned from legumes. Molecular cloning of resistance genes should facilitate studies on plant-pathogen interaction and ultimately facilitate genetic improvement of crop species.

DEDICATION

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PREFACE

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GENERAL INTRODUCTION

Anthracnose is a seed-borne disease of common bean (*Phaseolus vulgaris* L.) caused by the hemibiotrophic fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., that is found particularly in sub-tropical and temperate zones. Temperature and humidity are the most important environmental factors for anthracnose infection and expression of symptoms (Pastor-Corrales and Tu 1989). Spores, produced in acervuli, are surrounded by a water-soluble gelatinous matrix. Temperature ranging from 18 to 22°C is the optimum for spore formation and moderate rainfall and wind are essential for spore dissemination. The rainfall dissolves the water-soluble matrix and the wind carries the spores splashed by the rain. Relative humidity of at least 92% and free water on the foliage for 12 hours are required for spore germination (Tu 1982). These environmental conditions are related with the physiological needs of the pathogen.

Anthracnose symptoms initially appear on the lower surface of bean leaves and petioles as angular, dark, brick red lesions, which become dark brown to black. On the leaf veins, lesions appear as a slightly sunken canker. Lesions also appear on branches, main stems, and pods. Infection of pods produces flesh to rust-colored circular spots and the margin of the lesions is slightly raised. The center of the lesion contains a pink mass of spores in acervuli, and as the pod matures, these spore masses dry to gray, brown, or

black granulations. Plants in all stages of growth are subject to anthracnose. Infected seeds may display black cankers (Pastor-Corrales and Tu 1989).

Several control measures can be taken for control of anthracnose in the short-term. High priorities are given to selection of resistant cultivar, crop rotation, seed quality, and pesticide application. Crop rotation is important because spores can survive on plant debris for 12-18 months (Tu 1992). After harvest infected bean debris has to be incorporated into the soil, and beans should be in rotation with other crops for at least two years. Seed is the major source of primary inoculum. Thus, production of clean seed of cultivars resistant to the prevalent races of anthracnose in semi-arid regions and distribution to growers through a certified seed program are essential. *Colletotrichum lindemuthianum* can survive at least 5 years on pods and seeds that were air-dried and stored at 4°C (Tu 1983). Infected seed can be treated with fungicides such as benomyl or thiophanate methyl to effect partial control. Seed treatment can reduce disease incidence down to 5%. Use of fungicides can prevent or reduce anthracnose if they are applied early in the epidemic and coverage is thorough. Critical growth stages for fungicide application are 15 days after plant emergence and prior to flowering. Early infections may result in complete yield loss because secondary cycles of the disease are the most important for disease progress. Usually infection has to be monitored by the appearance of symptoms prior to scheduling a fungicide spray. Continued use of fungicides may favor the development of resistant biotypes of the pathogen (Pastor-Corrales and Tu 1989). To prevent disease spread, activity should be restricted in bean fields when plants are wet. The movement of animals, humans, and equipment can disperse spores, especially in fields planted under high-density (Pastor-Corrales and Tu 1989).

Complete yield loss can occur depending on weather conditions, susceptible cultivars, agronomic poor management of the crop, and disease pressure (Singh et al. 1991b). In the 1950's and early 1960's, anthracnose was a serious problem in Michigan causing severe yield loss. Efforts to control this disease were concentrated on the use of disease-free certified seed produced in semi-arid regions and the development of resistant cultivars through breeding programs. Although under control, anthracnose can always become a problem because of the pathogen variability and the potential for new races to appear. Thus, special attention should be paid at the appearance of symptoms in the field and attempts to detect races occurring in the field prior to onset of epidemic potential. In 1976, an outbreak occurred in Ontario that affected yield, seed quality, and marketability of beans (Tu et al 1984). The origin and appearance of new races remains unknown. Outbreaks of anthracnose have been reported in Michigan and Ontario in 1993 and new races of the causal organism, *C. lindemuthianum* have been identified (Kelly et al. 1994a). In 1997, the same race reappeared in two Michigan locations, Bay County and Montcalm County. Cyclic outbreaks are in part explained by the high variability of the pathogen and the occurrence of newly evolved or introduced races, favorable weather conditions, and grower laxity in crop rotation and seed selection.

Pathogen Variability

Isolates of *C. lindemuthianum*, known to exist in all continents, can be classified into distinct physiological races (Pastor-Corrales and Tu 1989). Although races of *C. lindemuthianum* were first described in the beginning of the 20th century (Barrus 1911, 1918), initially pathogenic variability was not thought to be great since sexual recombination is rare in the fungus population and dispersal over great distance is limited (Beebe and Pastor-Corrales 1991). However, in the last decade many new races have been identified in bean-growing countries throughout the world (Menezes and Dianese 1988, Pastor-Corrales and Tu 1989, Tu 1994, Kelly et al. 1994a). The full extent of the pathogen variability was difficult to assess because research groups in many countries have used local codes rather than the original Greek letters to identify races. Despite the tentative equivalency of described races in different countries, data collected using local hosts as differentials has limited the knowledge of the variability present within *C. lindemuthianum*, worldwide. A standard differential series of 12 bean cultivars of diverse origin and a binary system based on the position of each cultivar within this series were established (Table 1) to better understand and classify the variability and structure in *C. lindemuthianum* populations (Drifhout and Davis 1989, Pastor-Corrales 1991). The adoption of this standard procedure allows comparison and compilation of data from different research groups and a fuller characterization of the wide variability present in *C. lindemuthianum*. The re-characterization of races previously assigned Greek letters or local codes suggests an overestimation of variability reported for this pathogen.

Table 1. Anthracnose differential series, resistance genes, host gene pool, and the binary number of each cultivar.

Differential Cultivar	Host Genes[I]	Gene Pool[II]	Binary Number[III]
Michelite	--	MA	1
MDRK	*Co-1*	A	2
Perry Marrow	*Co-1*^{3}	A	4
Cornell 49242	*Co-2*	MA	8
Widusa	--	A	16
Kaboon	*Co-1*^{2}	A	32
Mexico 222	*Co-3*	MA	64
PI 207262	--	MA	128
TO	*Co-4*	MA	256
TU	*Co-5*	MA	512
AB 136	*Co-6*	MA	1024
G 2333	*Co-4*^{2}, *Co-5*, *Co-7*	MA	2048

[I] Host resistance genes. Not all resistance genes have been characterized.

[II] MA – Middle American gene pool, A – Andean gene pool (Singh et al 1991a).

[III] Binary number: 2^n, where n is equivalent to the place of the cultivar within the series. The sum of cultivars with susceptible reaction will give the binary number of a specific race (Pastor-Corrales 1991). *E.g.*, race 17 = virulent on Michelite (1) + Widusa (16).

Combining virulence and molecular analyses has led to a better understanding of the variability present in *C. lindemuthianum*. Molecular analysis of entire genomes can reveal the extent of variability in one species. Random Amplified Polymorphic DNA (RAPD) markers have been used to identify isolates of *C. lindemuthianum* (Balardin et al. 1997). Sequence analysis of specific regions of the genome, such as ribosomal DNA, is a powerful approach and complements studies on intraspecific diversity (Melotto et al. 1999). A specific set of primers, PN3 and PN10, was developed to amplify the rDNA region between the 18S and 28S genes in *C. lindemuthianum*, that includes Internal Trancribed Spacer (ITS) 1, 5.8S rRNA gene, and ITS 2 (Fabre et al. 1995). Polymerase Chain Reaction (PCR) amplification of this region produced a single reproducible 580 bp DNA fragment in all 57 isolates of *C. lindemuthianum* including *C. orbiculare* (Balardin et al. 1999). Digestion with the endonucleases *Hae* III and *Msp* I generated two patterns (Fabre et al. 1995, Sicard et al. 1997, Balardin et al. 1999). Polymorphism in rDNA does not appear to be linked to a specific virulence phenotype or structured with the geographic origin of races or host gene pool (Fabre et al. 1995). A variable level of genetic similarity among races and the lack of association of geographic origin with polymorphism in the rDNA region is clear evidence of the high level of molecular variability within *C. lindemuthianum*. In addition, insufficient support for parallel evolution of *C. lindemuthianum* and its host *P. vulgaris* is indicated by the high sequence similarity of the ITS region among Andean and Middle American races (Balardin et al. 1999). For instance, genetic distance among races belonging to different gene pools did not exceed the genetic distance among races of the same gene pool (Balardin et al. 1999).

Intra-race polymorphism, determined using RAPD and AFLP (Amplified Fragment Length Polymorphism) markers, was observed within several isolates of different races of *C. lindemuthianum* collected in different countries (Balardin et al. 1997, Sicard et al. 1997, Gonzales et al. 1998). For instance, isolates of race 65, characterized in the United States, showed a different RAPD pattern, whereas isolates from Brazil were monomorphic (Balardin et al. 1997). Isolates of race 0, collected in Mexico, showed distinct AFLP patterns (Gonzales et al. 1998) and isolates of races 7, 17, 31, and 73 from different countries showed polymorphism (Balardin et al. 1999). The intra-race polymorphism observed using molecular markers suggested a high level of molecular variability within *C. lindemuthianum* and emphasized the limitation of virulence analysis for assessing variability. The significance of such intra-race variability on pathogen population structure is poorly understood, but it suggests independent evolution of specific virulence phenotypes, such as race 73, in different geographic regions.

Host Genetic Resistance

Although unproven, resistance to anthracnose is likely to follow the gene-for-gene theory (Flor 1947). Resistance genes have been described in common bean (Melotto et al. 1999), but avirulence genes were never isolated or studied in *C. lindemuthianum*. Race-cultivar specificity is well known in the *P. vulgaris/C. lindemuthianum* pathosystem (Tu 1992) and hypersensitive reaction (HR) is characteristic of the incompatible interaction.

Morphological, biochemical, and molecular markers confirm the existence of a Middle American and an Andean gene pool within *P. vulgaris* (Gepts 1988, Singh et al. 1991, Haley et al. 1994b). These gene pools originated from divergence of the ancestral

population prior to domestication. Unique molecular and biochemical markers detected within wild bean populations from northern Peru and Ecuador, suggest the presence of an ancestral third gene pool within *P. vulgaris* (Kami et al. 1995). Host resistance genes are classified as either Middle American or Andean depending on the gene pool origin of the host cultivar. Races of *C. lindemuthianum* are similarly classified as either Middle American or Andean depending on the gene pool of the host cultivar for which each was isolated. Races of *C. lindemuthianum* virulent to Middle American hosts show greater diversity in pathogenicity by attacking germplasm from both gene pools, whereas races virulent to Andean hosts are mostly pathogenic on Andean germplasm (Beebe and Pastor-Corrales 1991, Balardin and Kelly 1998). Races alpha (race 17) and delta (race 23) were found to be more pathogenic on small-seeded, indeterminate (Middle American gene pool) cultivars, whereas races beta (race 130) and gamma (race 102) were pathogenic on large-seeded, determinate cultivars (Andean gene pool). Reciprocal selection of resistance genes in *P. vulgaris* and virulence genes in *C. lindemuthianum* might occur naturally.

To date, six independent dominant genes, which confer resistance to anthracnose in common bean, have been characterized. The Andean *A* gene reported by Burkholder (1918) and recently renamed *Co-1* (Kelly and Young 1996), and five Middle American genes, *Are* (*Co-2*) (Mastenbroek 1960), *Mexique 1 (Co-3)*, *Mexique 2 (Co-4)*, *Mexique 3 (Co-5)* (Fouilloux 1979), and *Co-6* (Young and Kelly 1996a).

The Andean *Co-1* gene was the first major gene utilized to develop anthracnose resistant cultivars of common bean (Burkholder 1923). Prior to 1978, the *Co-1* gene was used as the only source of resistance in navy beans grown in Michigan and Ontario. After

the appearance of race delta (race 23) in Ontario (Tu 1988) and the United States (Goth and Zaumeyer 1965), the Middle American *Co-2* gene, characterized in a black bean from Venezuela (Mastenbroek 1960), became the main source of anthracnose resistance in the North America. The identification of race 73 in Michigan and race alpha-Brazil (race 89) in Ontario, however, limited the further utilization of the *Co-2* gene (Kelly et al. 1994a, Tu 1994). Combining the *Co-1* and *Co-2* genes in single cultivars was suggested as the best short-term protection against all known races currently present in North America (Kelly et al. 1994a, Young and Kelly 1996a).

The *Co-2* gene was the first predominant source of resistance to be used worldwide. The appearance of races 31, 63, and 89 virulent to the *Co-2* gene, however, emphasized the need for new sources of resistance (Kruger et al. 1977). In France, new resistance genes *Co-3 (Mexique I)*, *Co-4 (Mexique II)* and *Co-5 (Mexique III)* were characterized in a collection of bean germplasm from Mexico (Fouilloux 1979). The *Co-4* and *Co-5* genes conferred resistance to current races virulent to the *Co-2* gene, whereas the *Co-3* gene was overcome by race 89. Virulence of *C. lindemuthianum* has been monitored in different countries, and evolving races have continually overcome resistant germplasm. Durability of resistance depends on how efficiently resistance against the newest race can be incorporated into commercial germplasm as new predominant virulence phenotypes evolve within the pathogen population.

Breeding for Anthracnose Resistance

For long-term control of bean anthracnose, development of resistant cultivars is the only practical approach. Sources of anthracnose resistance in different bean genotypes are well known. There is resistance in navy, black, and kidney bean germplasm. Other bean classes such as pinto, great northern, small reds, pinks, and cranberries have little or no resistance to anthracnose. Resistance from Andean kidney beans was incorporated in Middle American bush navy beans including Sanilac, Seafarer, Gratiot, Kentwood, Fleetwood and black beans such as Raven and is still effective in North America after 20 years. New races, however, have been isolated from Isabella (light red kidney), Blackhawk (black) and Aztec (pinto) that overcome this resistance (Kelly et al. 1994a). Cultivars, such as Isles and Newport (Kelly et al. 1994b, 1995) that are resistant to all current races in Michigan, have been recently developed. These cultivars have combined sources of resistance from kidney and black beans.

The information on variability in *C. lindemuthianum* and the specialization of specific races on one host gene pool is invaluable in breeding for resistance. Numerous studies have indicated that resistance to *C. lindemuthianum* in common bean is controlled by major genes acting singly (Young and Kelly 1996a), as duplicate or complementary factors (Cardenas et al. 1964, Muhalet et al. 1981) or as members of an allelic series (Fouilloux 1979, Young et al. 1998, Melotto and Kelly 1999). The most resistant differential cultivar, G 2333, first thought to possess two dominant genes (Pastor-Corrales et al. 1994), was shown to possess three independent genes *Co-4*^{2}, *Co-5,* and *Co-7* (Young and Kelly 1997, Young et al. 1998). Pyramiding major resistance genes may be the most appropriate breeding strategy for long-term resistance in *P. vulgaris*

(Young and Kelly 1997). Knowledge of gene complementarity has been suggested as a strategy to improve the efficiency of pyramiding genes for durable resistance (Duvick 1996). If pathogens were specialized on one of the host gene pools, pyramiding resistance genes from different gene pools may provide a more durable anthracnose resistance (Young and Kelly 1997). For instance, the incorporation of Andean resistance genes in bean breeding populations in Honduras where Middle American races predominate; or Middle American resistance genes in germplasm in the Dominican Republic where Andean races prevail could result in more durable resistance in each country (Balardin and Kelly 1998). The reduced virulence of isolates from Andean regions compared to isolates from Middle American regions suggests that deployment of resistance genes between gene pools should extend host resistance (Gepts 1988, Balardin and Kelly 1998).

Despite the large number of studies indicating that resistance to anthracnose is controlled by either major dominant (Kelly and Young 1996a) or recessive resistance genes (Cardenas et al. 1964, Muhalet et al. 1981, Alzate-Marin et al. 1997), other resistance mechanisms have been reported (Pastor-Corrales et al. 1985). The cultivar, ICA Llanogrande is susceptible to anthracnose in seedling assays, but is resistant to the same isolates under field conditions in widely different geographic regions of South America and Africa. Under controlled greenhouse testing, plants of ICA Llanogrande exhibit progressively greater resistance with age, but the mechanism or mode of inheritance is unknown (Beebe and Pastor-Corrales 1991). Under field conditions, the Brazilian cultivar Rio Negro, which carries the defeated *Co-2* gene, exhibits resistance against races of *C. lindemuthianum* to which it is known to be susceptible in seedling

assays. The possibility of combining nonspecific resistance with major gene resistance for anthracnose may, therefore, exist (Beebe and Pastor-Corrales 1991).

Marker Assisted Selection

Selection for cultivars carrying pyramided genes is most effective when indirect selection assisted by the use of molecular markers tightly linked to the gene of interest is practiced. The efficient pyramiding of epistatic resistance genes with the aid of linked molecular markers has been demonstrated in common bean (Kelly and Miklas 1998). Marker-assisted selection (MAS) can only be practiced if robust and reliable markers are available (Melotto et al. 1996). Choice of the marker for MAS mostly depends on the ease of the analysis. PCR-based markers are the common choice because only a small amount of DNA is needed in addition to be cost-effective. More elaborate markers such as RFLP, AFLP, usually require well-equipped laboratories and are time-consuming. However, these markers, once identified, can be partially sequenced to design specific primers and the marker can be transformed into a sequence characterized amplified region (SCAR) marker. SCAR markers are usually dominant in nature and a quick minus/plus assay can be applied to detect the PCR product. As suggested by Gu et al. (1995), ethidium bromide is added to the amplification reaction and strong fluorescence is observed in samples containing amplified DNA, whereas weak fluorescence is observed in samples in which no fragment is present. Direct staining with ethidium bromide can be used in breeding programs when genotyping a large number of samples is required, to facilitate the development of more disease-resistant cultivars using marker assisted breeding (Gu et al. 1995, Melotto et al. 1996).

Genetic maps are the source of markers used to locate single or quantitative trait loci (QTL; Adam-Blondon et al. 1994). Morphological and agronomic traits present in the bean linkage map include the *I* gene for BCMV resistance, flower and seed color genes (Vallejos et al. 1992, Nodari et al. 1993a), a seed size gene (Vallejos and Chase 1991), the *Co-2* gene for anthracnose resistance, the *Ms8* gene conferring male sterility, the *SGou* gene for pod-shape character (Adam-Blondon et al. 1994), and the QTLs related to common bacterial blight and nodule number (Nodari et al. 1993b). Three linkage maps of common bean are available (Vallejos et al. 1992, Nodari et al. 1993a, Adam-Blondon et al. 1994) based on RFLPs, RAPD, isozymes, proteins, and phenotypic markers, but none of these maps is completely saturated and the number of linkage groups does not correspond to the number of bean chromosomes. In a recent publication (Freyre at al. 1998), the three bean linkage maps were integrated to form a complete, more accurate map of common bean based on different types of markers. Now the number of linkage groups corresponds to the number of chromosomes in common bean. The current bean linkage map still has limited genetic information on agronomic traits useful for breeders. However, breeders who want to tag a specific locus may use the markers available in the map to screen their populations.

Many loci important for breeding have been tagged using bulked segregant analysis (Michelmore et al. 1991). This technique allows rapid identification of tightly linked markers useful for MAS. For instance the *I* gene has been tagged with the RAPD marker $OW13_{690}$ (Haley et al. 1994a), which was later transformed into the SCAR marker SW13 (Melotto et al. 1996). Markers of the bean genome must be tested in a collection of common bean genotypes representing both Andean and Middle American

gene pools (Singh et al. 1991a) because markers may not be polymorphic across gene pools or races within gene pools. The presence or absence of the SW13 marker corresponded to the presence or absence of the *I* gene indicating broad utility of this marker across bean gene pools. In previous germplasm surveys of common bean, certain RAPD markers have been shown to be very useful as tools for indirect selection in populations derived from both gene pools (Haley et al. 1994c, Young and Kelly 1996b), whereas the application of other markers was restricted to a specific gene pool (Miklas et al. 1993) or to a certain race within a gene pool (Haley et al. 1993).

Map-Based Cloning of Resistance Genes

Knowledge of gene location in the plant genome allows the isolation of that particular gene and in depth study of resistance gene function. Isolation of disease resistance genes is not only important for direct transformation of susceptible cultivars, but also to understand the mechanisms of resistance, which may lead to novel approaches for disease control. Several approaches exist to clone disease resistance genes. In the early 1990's, the *Cf-9* gene, which confers resistance to *Cladosporium fulvum* in tomato, was isolated by transposon tagging (Jones et al. 1994). An alternative approach is to use a tightly linked molecular marker for chromosome walking to the gene. Map-based cloning has been used to isolate the *Pto* gene of tomato (Martin et al. 1993). Since then, many other resistance genes have been cloned from different plant taxa. Some examples are *Xa21* of rice (Song et al. 1995), *RPS2* and *RPM1* of Arabidopsis (Bent et al. 1994, Mindrinos et al. 1994, Grant et al. 1995), *N* of tobacco (Whitham et al. 1994), and *L6* of flax (Lawrence et al. 1995).

The classic 'gene-for-gene' theory proposed by Flor (1947) explains that a complementary pair of dominant genes, one in the host and another in the pathogen, is necessary for resistance to occur. This model suggests a receptor-like function for the resistance gene product, whereas the avirulence gene product would be the ligand. After 50 years, Flor's theory has been shown to exist at the molecular level in the tomato/*Pseudomonas syringae* interaction. The Pto protein directly binds to the avrPto protein triggering the defense reaction (Scofield et al. 1996, Tang et al. 1996). Analysis of proteins encoded by cloned resistance genes has revealed a striking similarity and these proteins appear to have key role in plant/pathogen interaction (Bent 1996). Independent of the plant taxa or the pathogen involved, resistance gene-encoded proteins possess a few conserved functional domains, leucine rich repeats (LRR), nucleotide binding sites (NBS), leucine zippers (LZ), and protein kinases (Hammond-Kosack and Jones 1997). Each resistance gene may encode one or more domains. For instance, *Pto* encodes a serine threonine kinase (STK) and *RPM1* encodes a three-domain protein with LRR, NBS and LZ consensus sequences. Leucine-rich repeat proteins could be responsible for recognition specificity as it can bind corresponding ligands from the pathogen. Nucleotide binding sites, LZ and kinases may be involved in signal transduction pathways to activate gene transcription. These domains are usually involved in activating other proteins, which may trigger a chain of events leading to defense response (Bent 1996, Hammond-Kosack and Jones 1997).

Based on the characteristics of disease resistance genes, several research groups have been using the DNA sequence information of conserved regions to map and isolate disease resistance related genes from other plant organisms. In common bean and

soybean, resistance genes analogs (RGAs) were identified using primers specific to conserved regions of known resistance genes and mapped close to known disease resistance locus (Kanazin et al. 1996, Yu et al. 1996, Geffroy et al. 1998, Rivkin et al. 1999). Mapping of RGAs has been the only 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 known resistance locus limiting their value in chromosome walking to the gene. Additional approaches, such as map-based cloning are still needed to isolate resistance gene candidates in crop species.

GENERAL OBJECTIVES

Genetic inheritance of anthracnose resistance is well studied. Major dominant resistance genes are involved in controlling this devastating disease and have been extensively used in breeding programs worldwide. However, the fungus *C. lindemuthianum*, the causal agent of anthracnose, is highly variable and new pathogenic types that overcome cultivar resistance continue to evolve. Therefore, breeders always need new sources of resistance as well as a fast and reliable approach to incorporate this resistance into elite breeding lines. In this dissertation, the objectives were to characterize new Andean sources of anthracnose resistance, to identify molecular markers (RAPD and SCAR) tightly linked to the *Co-4* locus, and use these markers as a starting point for chromosome walking towards the gene.

In order to successfully achieve these objectives, the research project was divided into three main sections, which are described in each chapter of this dissertation. The objective in Chapter 1 was to study the genetic inheritance of the anthracnose resistance present in the bean cultivars Kaboon and Perry Marrow and describe the relation of this resistance to other independent loci conditioning resistance to anthracnose. The objectives in Chapter 2 were to find a RAPD marker tightly linked to the *Co-4*^{2} gene present in the bean breeding line SEL 1308 and transform the marker into a SCAR. This molecular marker would be used to dissect the genetic resistance of G 2333, which carries three anthracnose resistance genes, *Co-4*^{2}, *Co-5*, and *Co-7*. The first two genes have been isolated in two different bean lines and the *Co-7*, however, could not be isolated because it is hypostatic to the *Co-4*^{2} gene. Tagging the *Co-4*^{2} was the only approach to further characterize the *Co-7* gene. The objectives in Chapter 3 were to fine map the *Co-4*^{2} gene using a large F_2 segregating population and clone DNA sequences involved in disease resistance. The *Co-4*^{2} gene is a valuable resistance gene as it confers resistance to many highly virulent races of anthracnose. Once cloned, *Co-4*^{2} could be used to transform susceptible bean cultivars to rapidly improve genetic resistance to anthracnose.

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CHAPTER ONE

AN ALLELIC SERIES AT THE *Co-1* LOCUS CONDITIONING RESISTANCE TO ANTHRACNOSE IN COMMON BEAN

ABSTRACT

In this study, the genetic resistance of the Andean bean cultivar Kaboon and its relation to other sources of anthracnose resistance in common bean was characterized. Based on the segregation ratio (3R:1S) observed in two F_2 populations it is concluded that Kaboon carries one dominant gene conferring resistance to races 7 and 73 of *Colletotrichum lindemuthianum*. This gene in Kaboon is independent from the *Co-2* gene and is an allele of the *Co-1* gene present in Michigan Dark Red Kidney (MDRK). Support for allelism is based on the differential reaction of these genotypes to a range of anthracnose races. Therefore, the symbol $Co\text{-}1^2$ for the major dominant gene in Kaboon is proposed. Among the *Co* anthracnose resistance genes characterized in common bean, the *Co-1* is the only gene of Andean origin. When inoculated with a less virulent race, the Andean race 5, segregation ratio in the F_2 progeny of Cardinal and Kaboon was 57R:7S ($p=0.38$) indicating that Kaboon may have other dominant resistance genes with complementary mode of action, since Cardinal possesses no known genes for anthracnose resistance. Therefore, at least two of the three dominant genes segregating in this cross must come from Kaboon. Perry Marrow, a second Andean genotype which conditions

resistance to a different group of races, appears to have another resistance allele at the *Co-1* locus and the gene symbol *Co-1*^{3} was assigned. In R x R crosses between Perry Marrow and MDRK or Kaboon, no susceptible F_2 plants were found when inoculated with race 73. These findings have major implication in developing breeding strategies for durable anthracnose resistance. If different alleles occur at the same locus, breeders may have limited sources of Andean resistance genes available for gene pyramiding and many of these genes may reside at the *Co-1* locus.

INTRODUCTION

The existence of Middle American and Andean gene pools within common bean, *Phaseolus vulgaris* (L.) has been demonstrated based on morphological traits, phaseolin types, isozymes, molecular markers, and lethality of Middle American/Andean hybrids (Gepts 1988, Singh et al. 1991, Haley et al. 1994). Unique molecular and biochemical markers detected within wild bean populations from northern Peru and Ecuador, suggest the presence of a third ancestral gene pool within *P. vulgaris* (Kami et al. 1995). Likewise, host resistance genes and races of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cav., the causal agent of anthracnose in common bean, are classified as either Middle American or Andean depending on the gene pool origin of the host cultivar. Races of *C. lindemuthianum* virulent to Middle American hosts show greater diversity in pathogenicity by attacking germplasm from both gene pools, whereas races virulent to Andean hosts are mostly pathogenic on Andean germplasm (Balardin and Kelly 1998, Balardin et al. 1997). In general, Middle American hosts are more resistant to Andean races and Andean hosts are resistant to Middle American races. For instance, the *Co-1* gene (Andean) conditions resistance to race 73 (Middle American) and the *Co-2* gene (Middle American) conditions resistance to race 7 (Andean). Combining *Co-1* and *Co-2* genes in the same cultivar, is the best strategy to control these two highly virulent races currently present in North America. Broad-based resistance to bean anthracnose, therefore, can be achieved if both Andean and Middle American genes are combined in the same cultivar (Young and Kelly 1996ab). However, Andean sources of anthracnose resistance have not been studied as extensively as Middle American sources. There is

limited information available on the independence of the individual genes present in Andean cultivars or on the relationship of these genes to previously characterized anthracnose resistance genes. To date, only one Andean anthracnose resistance gene has been described, the *Co-1* gene (Burkholder 1918), which is found in the kidney bean cultivars Michigan Dark Red Kidney and Montcalm (Young and Kelly 1997).

New sources of anthracnose resistance must always be sought because of the high variability in the pathogen population and the occurrence of newly evolved virulent races. In a recent study, Balardin and Kelly (1997) found that the Andean differential cultivar Kaboon is resistant to all but two isolates of *C. lindemuthianun* collected from bean fields throughout the Americas. Muhalet et al. (1981) suggested that Kaboon carries one dominant and two complementary factors conferring resistance to race beta, one dominant complementary factor conferring resistance to race gamma, and two complementary factors conferring resistance to race delta. These authors also suggested that Kaboon does not carry the dominant *Co-2* gene. Kaboon is susceptible only to races 55 (lambda), 63 (iota), 99 (theta), 102 (gamma), and 357, from among races presently characterized (Tu 1994, Balardin and Kelly 1997).

There is no information available on the independence of the individual genes present in the Andean differential cultivar Kaboon and Perry Marrow or on the relationship of these genes to other previously characterized resistance genes. In this chapter, the genetic characterization of anthracnose resistance present in Kaboon and its relation to other Andean cultivars such as Perry Marrow is discussed. Evidence for existence of an allelic series at the *Co-1* locus in common bean is presented.

MATERIAL AND METHODS

Genetic material. Several segregating populations were developed to determine the inheritance and independence of the anthracnose resistance genes present in Kaboon. Kaboon is a large-seeded white cultivar of Andean origin and was used as the male parent of all populations. The female parents were the Andean genotypes Cardinal, Perry Marrow and Michigan Dark Red Kidney (MDRK) and the Middle American genotypes Cornell 49-242 and Flor de Mayo M38 (Table 1). Cardinal possesses no known gene for anthracnose resistance, whereas MDRK carries the *Co-1* gene and Cornell 49-242 carries the *Co-2* gene. Perry Marrow and Flor de Mayo M38 possess resistance to certain races of *C. lindemuthianum*, however anthracnose resistance gene(s) have not been previously described in these cultivars. Perry Marrow was crossed with MDRK to assess the genetic independence of the resistance genes present in these two cultivars. F_2 populations and their respective $F_{2:3}$ families were used to determine inheritance and independence of Andean genes for anthracnose resistance. For the test of allelism, six F_2 populations were developed using cultivars carrying independently characterized genes MDRK (*Co-1*), Cornell 49-242 (*Co-2*), Mexico 222 (*Co-3*), SEL 1308 (*Co-4*^{2}), SEL 1360 (*Co-5*), and Catrachita (*Co-6*) (Young and Kelly 1996a).

Races of *C. lindemuthianum* and inoculum preparation. Three races of *C. lindemuthianun* were chosen based on the differential phenotypic reaction observed in parental genotypes (Table 2), the Andean race 5 (collected in Peru) and race 7 (ATCC 96390), and the Middle American race 73 (ATCC 96512). Race 7 is highly virulent on

Andean cultivars such as Perry Marrow and MDRK, whereas race 73 is highly virulent on Middle American cultivars. Kaboon is resistant to all races used in this study. Reaction of the parents to each race is described in Table 1. Identification of each race was confirmed by the reaction observed on the cultivar differential series (Melotto et al. 1999). Each race was grown from monosporic cultures maintained in fungus-colonized filter papers stored at –20° C. To prepare the inoculum, fungal culture was grown by using infected tissue incubated under sterile conditions on modified Mathur's culture medium containing dextrose (8g/L), $MgSO_4$. 7 H_2O (2.5g/L), KH_2PO_4 (2.7g/L), neopeptone (2.4g/L), yeast extract (2g/L), and agar (16g/L). Spores were re-plated and used as the source of inoculum. All cultures were incubated for 10 days in complete darkness at room temperature. Spore suspensions were prepared by flooding culture plates with 0.01% Tween 20 in sterile distilled water and scraping the culture surface. Inoculum concentration was adjusted to 1.0×10^6 spores/ml using a hemacytometer.

Disease phenotypic evaluation. Spore suspension of the pathogen was evenly sprayed on 10 day-old seedlings at the primary leaf stage. After inoculation, seedling were maintained in a mist chamber with relative humidity higher than 95% for 48 hours at 22-25° C. Symptoms were evaluated seven days after inoculation. Plants were rated as resistant when no lesions were observed and rated as susceptible when enlarged lesions were observed on the lower leaf surface and stem. Resistant and susceptible cultivars used in this study presented extreme disease reactions to the chosen races.

Segregation ratios and statistical analysis. Segregation ratios tested in the F_2 populations were 3R:1S for a single dominant gene, 15R:1S for two independent

dominant genes, and 57R:7S for a single dominant gene plus two complementary dominant genes. In the $F_{2:3}$ families, the segregation ratio tested was 1:2:1 to discriminate between homozygous and heterozygous resistant F_2 plants. All observed ratios were compared to the expected ratios using the Chi-square test for goodness of fit. Probabilities (P values) are shown in Tables 3 and 4.

RESULTS

Gene pool origin of bean cultivars and their disease reaction to the races used in this study are presented in Table 1. Cornell 49-242 and Flor de Mayo M38 were used to determine the independence of the Andean gene(s) present in Kaboon from Middle American genes. The Andean bean cultivars Cardinal and MDRK were used to determine the number of genes present in Kaboon, whereas the cultivars Perry Marrow and MDRK were used to determine the resistance relationship of genes in Kaboon to other Andean genes. All crosses, generation tested, and races used for the allelism test or genetic inheritance of segregating genes, are presented in Table 2. In all inoculation experiments, no intermediary reaction was observed. Susceptible plants died and resistant plants showed no symptoms after seven days of inoculation.

The segregation ratio 3R:1S ($p=0.34$) observed in the Cardinal x Kaboon F_2 population (Table 3) indicates that Kaboon carries one dominant gene conferring resistance to race 7 of *C. lindemuthianum*. In a mixed inoculation with races 7 and 73, the same 3:1 ratio was supported, suggesting that the same single dominant gene conditions resistance to these two widely dispersed Andean and Middle American races. To determine whether Kaboon has other resistance genes, the F_2 progeny of Cardinal and Kaboon was inoculated with a less virulent race, the Andean race 5. The observed segregation ratio 57R:7S ($p=0.38$) indicates that Kaboon may have other dominant resistance genes with complementary mode of action, since Cardinal possesses no known genes for anthracnose resistance. Therefore, at least two of the three dominant genes segregating in this cross must come from Kaboon.

Table 1.1 Gene pool origin, described anthracnose resistance genes, and disease reaction of bean cultivars inoculated with different races of *C. lindemuthianum*.

			Races of *C. lindemuthianum*		
Bean Cultivar	Gene Pool[a]	Known gene	5	7	73
Cornell 49-242	MA	*Co-2*	R[b]	S	R
FM 38	MA	unknown	R	R	R
Cardinal	A	none	S	S	S
Perry Marrow	A	*Co-1*^{3}	S	R	S
MDRK	A	*Co-1*	R	R	S
Kaboon	A	*Co-1*^{2}	R	R	R

[a] A= Andean; MA= Middle American (Singh et al. 1991).

[b] R= resistant reaction, S= susceptible reaction.

Table 1.2 Crosses, races, disease reaction, and generations used for the genetic characterization of anthracnose resistance in Kaboon.

Cross	Race	Reaction[a]	Generation[b]
MDRK x Kaboon	73	R x R	P, F_2
MDRK x Kaboon	7	S x R	P, F_2, $F_{2:3}$
Cornell 49-242 x Kaboon	7	R x R	P, F_2
Cardinal x Kaboon	5	S x R	P, F_2
Cardinal x Kaboon	73	S x R	P, F_2
Cardinal x Kaboon	7 + 73	S x R	P, F_2
Flor de Mayo M38 x Kaboon	73	R x R	P, F_2
Perry Marrow x Kaboon	73	R x R	P, F_2
MDRK x Perry Marrow	73	R x R	P, F_2

[a]R = resistant reaction and S = susceptible reaction.

[b]Parental, F_2 plants, $F_{2:3}$ families, respectively.

Upon inoculating the MDRK x Kaboon F_2 population with race 7 (S x R cross), segregation in the F_2 generation again suggested the presence of a single dominant gene in Kaboon conferring resistance to race 7. These data were confirmed in progeny tests in the $F_{2:3}$ families, where progeny genotypes segregated 1:2:1 (p=0.57). Allelism test was conducted in the same F_2 population using race 73 (R x R cross). No segregation was observed in 174 F_2 individuals inoculated, suggesting that the gene in Kaboon, which conferred resistance to races 7 and 73, was allelic to the *Co-1* gene in MDRK. Clearly the genes have to be allelic since one (MDRK) conditioned resistance to only race 73 while the other (Kaboon) conditioned resistance to both races 7 and 73. Support for allelism is based on the differential reaction of these genotypes to a range of anthracnose races.

Independence of the gene present in Kaboon was tested in the F_2 population derived from a cross between Cornell 49-242 and Kaboon. Inoculation with race 7 (R x R cross) resulted in a segregation of 15:1 (p=0.51) suggesting the presence of two independent dominant genes, one the *Co-2* gene in Cornell 49-242 and the other an allele of the *Co-1* gene (Table 4). With the confirmation that these loci were independent and that the gene in Kaboon is allelic to the *Co-1* gene, no further allelic tests were conducted with other independent loci (*Co-3* to *Co-6*). It is proposed to name the gene which conditions resistance to race 7 and 73 in Kaboon as the $Co\text{-}1^2$ allele. The other complementary dominant genes in Kaboon do not appear to offer a greater level of resistance to more virulent races of anthracnose. There was no evidence for recessive resistance in any of the crosses studied.

The anthracnose resistance gene(s) present in the bean cultivar Flor de Mayo M38 have not been characterized genetically. This cultivar is resistance to races 7 and 73. F_2

populations derived from a cross with Kaboon (R x R) was inoculated with race 73. A segregation ratio 15:1 (p=0.7) was observed, indicating that each of the parents carry a dominant independent resistance gene. This result supports the independence of the *Co-1²* allele (Kaboon) from other Middle American resistance genes. The anthracnose resistance present in Flor de Mayo 38 could have originated from its Middle American background (Acosta-Gallegos et al. 1995).

The Andean cultivar Perry Marrow was crossed with MDRK (*Co-1*) and Kaboon (*Co-1²*) for allelism test. No segregation was observed in 152 or 143 F_2 individuals from each population (Table 4). These three Andean cultivars have different levels of resistance to anthracnose. For instance, at least seven races including races 5, 357, 1165 attacks Perry Marrow but not MDRK (Balardin et al. 1997). Therefore, the *Co-1* allele present in Perry Marrow is different from the one in MDRK. In addition, Perry Marrow does not possess the *Co-1²* allele because Kaboon is more resistant than Perry Marrow (Table 1). These results suggest the presence of another allele at the *Co-1* locus. The gene symbol *Co-1³* was assigned to the major gene present in Perry Marrow that confers resistance to race 73.

Table 1.3 Observed and expected segregation ratios of progenies for their reaction to *C. lindemuthianum* in SxR crosses. Kaboon (K) is the male parent of all crosses.

		Phenotypic Evaluation in the F_2 (no. plants)				Phenotypic Evaluation in the $F_{2:3}$ (no. plants)			
Cross	Race	Exp ratio	R-	rr	P value	RR	Rr	rr	P value
MDRK x K	7	3:1	80	22	0.42	21	55	25	0.57
Cardinal x K	5	57:7	60	10	0.38				
Cardinal x K	73	3:1	125	49	0.34				
Cardinal x K	7+73	3:1	70	28	0.41				

Table 1.4 Allelism test for the genetic characterization of the resistance to *C. lindemuthianum* in Kaboon and Perry Marrow cultivars.

		Phenotypic Evaluation in the F_2 (no. plants)			
Cross	Race	R-	rr	Expected ratio	P value
MDRK x Kaboon	73	174	0	all R	1.0
Cornell 49-242 x Kaboon	7	55	5	15:1	0.51
Flor de Mayo M38 x Kaboon	73	90	5	15:1	0.70
Perry Marrow x Kaboon	73	152	0	all R	1.0
MDRK x Perry Marrow	73	143	0	all R	1.0

DISCUSSION

In this chapter, genetic evidence for the occurrence of different alleles at the *Co-1* locus, which conditions resistance to anthracnose, is presented. Therefore, the symbol *Co-1*^{2} is proposed for the major dominant gene present in the Andean bean cultivar Kaboon, which condition resistance to races 7 and 73. The symbol *Co-1*^{3} is proposed for the resistance gene present in Perry Marrow. Occurrence of more than one resistance allele at *Co* loci is not unique. Two different resistance alleles at the independent *Co-3* locus, for instance, confer resistance to different races of the pathogen *C. lindemuthianum* (Fouilloux 1979). In a more recent study (Young et al. 1998), a second allele in SEL 1308 at the *Co-4* locus was identified and it is the broadest-based source of resistance among all *Co* genes identified to date (Balardin and Kelly 1998). As new sources of resistance are identified and characterized, there is increasing evidence for new alleles instead of new resistance genes. These results alert us to the need for allelism tests every time a new resistance source is recognized. All characterized anthracnose resistance genes have been isolated in different bean lines, which facilitates allelism tests without the interference of other epistatic genes.

In two published studies (Cardenas et al. 1964, Muhalet et al. 1981) reverse of dominance in populations segregating at the *Co-1* locus was observed. Change of dominance was not adequately addressed in these papers. Depending on the genotypes involved in those crosses the reverse of dominance observed could be explained by a multiple allelic series at that specific locus. For instance, if a race such as beta (race 130) possesses multiple virulence genes such that it is virulent to some alleles of the resistance

genes but not all the alleles at the *Co-1* locus, either 3R:1S or 1R:3S ratios would be observed depending on the specific combinations of alleles segregating in the different crosses. In an R x R cross between the cultivar Tuscola and Kaboon, two *Co-1* alleles segregated and no susceptible plants were found (Muhalet et al. 1981). When Tuscola was crossed with Montcalm (*Co-1* gene) and the F_2 population was inoculated with the same race 130, a 1R:3S ratio was observed. These results indicate that Montcalm carries a *Co-1* allele dominant over the one in Tuscola. Race 130 defeats the Montcalm allele but not the Tuscola allele. Clearly a complex multiple allelic series exists at the *Co-1* locus.

Breeding for durable anthracnose resistance has focused on gene pyramiding. Since resistance is conferred mostly by major dominant genes and each gene controls certain races of the pathogen, the logical choice for broad-based resistance is to combine genes based on their complementary reaction to the pathogen. Gene complementarity for durable resistance is achieved by combining Andean and Middle American genes in the same cultivar. Andean genes are most useful to improving Middle American cultivars and in some locations breeders may not have many choices of Andean sources to improve local bean cultivars. Our findings indicate that many Andean sources are allelic and the major locus for anthracnose resistance is *Co-1*. Other cultivars such as A-193 (Mendoza-Herrera et al. 1999), Perry Marrow, MDRK, Tuscola, Seafarer, Montcalm appear to possess alleles at *Co-1* locus as indicated by allelism tests, range of resistance, and DNA analysis (Young and Kelly 1996c). These cultivars do not possess the $OF10_{530}$ RAPD marker, which is linked in repulsion to the *Co-1* locus (Young and Kelly 1997). The choice of Andean resistance is among alleles not genes as they confer resistance to different races. Breeders will have to identify local races and deploy the appropriate

resistance allele to control them. For instance, the *Co-1*^{2} gene present in Kaboon is the choice for the bean breeding program in Michigan as it confers resistance to both Andean and Middle American races of anthracnose recently identified at that location.

Increasing evidence suggests that anthracnose resistance genes, once characterized as single independent dominant genes, are complex loci and occur as allelic series and multigene families in the bean genome. These recent findings have major implications not only in breeding programs as discussed above, but also in agricultural biotechnology. Two independent anthracnose genes, *Co-2* and *Co-4* have been studied at the molecular level and they appear to be part of complex loci (Geffroy et al. 1998, Young et al. 1998). These loci most often harbor multiple open reading frames and molecular cloning may be laborious and time-consuming. If geneticists choose to use cloned gene(s) for the transformation of susceptible cultivars, multiple genes must be used to achieve the desired level of resistance.

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CHAPTER TWO

MARKER-ASSISTED DISSECTION OF THE OLIGOGENIC ANTHRACNOSE RESISTANCE IN THE COMMON BEAN CULTIVAR, G 2333

ABSTRACT

Two independently assorting dominant genes conditioning resistance to bean anthracnose were previously identified in an F_2 population derived from the highly resistant bean differential cultivar, G 2333. One gene was allelic to the *Co-4* gene in the differential cultivar TO and was named *Co-4²*, whereas the second gene was assigned the temporary symbol *Co-7* until a complete characterization with other known resistance genes can be conducted. A RAPD marker linked to the *Co-4²* allele was identified. $OAS13_{950}$ and *Co-4²* allele co-segregated with no recombinants in two segregating populations of 143 F_2 individuals. A SCAR marker (SAS13), developed from the $OAS13_{950}$ RAPD marker, was dominant and polymorphic similar to the original RAPD, and supported the tight linkage between the marker and the *Co-4²* allele. The markers were present in germplasm with known resistance alleles at the *Co-4* locus. The presence of the markers in two other differential cultivars, not previously characterized, and in four navy bean cultivars suggests the existence of a gene family for anthracnose resistance at or near the *Co-4* locus. Since the *Co-7* gene was present only in germplasm which also possessed the *Co-4²* and *Co-5* genes, the SAS13 marker was used in combination with standard inoculation techniques to identify

F_3 lines in which the *Co-7* gene was homozygous and the *Co-4²* allele was absent. A similar strategy of marker assisted dissection is proposed to identify resistant lines in which the *Co-5* gene is absent and the *Co-7* gene is present by selecting against the $OAB3_{450}$ marker, shown previously to be linked to the *Co-5* gene. These genes cannot be distinguished using traditional screening methods since all current races of the pathogen virulent to the *Co-5* gene are avirulent to the *Co-4²* and *Co-7* genes. The use of molecular markers, tightly linked to resistance genes, to facilitate the identification of an uncharacterized resistance gene for which no discriminating race of the pathogen is known, is described.

INTRODUCTION

One of the most severe, widespread diseases of common bean (*Phaseolus vulgaris* L.) is anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lambs.-Scrib. (Pastor-Corrales et al. 1995, Schwartz 1991, Tu 1992). Genetic variability of *C. lindemuthianum* has been observed in different parts of the world where beans are grown (Beebe and Pastor-Corrales 1991, Pastor-Corrales and Tu 1989), and many races of the pathogen have been characterized (Balardin et al. 1997, Rava et al. 1993, Garrido-Ramirez and Romero-Cova 1989, Pastor-Corrales and Tu 1989, Menezes and Dianese 1988, Yerkes and Teliz-Ortiz 1956). Due to the highly variable nature of the pathogen and the continual emergence of new races, genetic resistance in the host is not durable. Currently, races of *C. lindemuthianum* are classified by inoculating isolates onto a universal set of 12 differential cultivars. Races are assigned a cumulative numerical value for each susceptible differential cultivar based on an established binary system (CIAT 1988, Pastor-Corrales 1991). Only six of the 12 differential cultivars have been genetically characterized for anthracnose resistance: Michigan Dark Red Kidney, *Co-1* gene (Burkholder 1918, Young and Kelly 1997); Cornell 49242, *Co-2* gene (Mastenbroek 1960); Mexico 222, *Co-3* gene; TO, *Co-4* gene; TU, *Co-5* gene (Fouilloux 1979); and AB 136, *Co-6* gene (Young and Kelly 1996a). There is limited evidence that some of the remaining six differentials, such as Perry Marrow and PI 207262, may carry more than one resistance factor (Cardenas et al. 1964, Muhalet et al. 1981, Gonçalves-Vidigal et al. 1997). For instance, using race 521, Pastor-Corrales et al. (1994) demonstrated that anthracnose resistance in differential cultivar G 2333 was controlled by two unknown resistance genes. In addition to these two unknown genes, Young and Kelly

(1996a) showed that G 2333 carried also the *Co-5* gene, which was not detected by Pastor-Corrales et al. (1994) since race 521 is virulent to the *Co-5* gene. The presence of more than one gene in G 2333 may account for its broad resistance to all known races of *C. lindemuthianum* (Balardin et al. 1997, Pastor-Corrales et al. 1994, Schwartz et al. 1982). If resistance in G 2333 is conditioned by three resistance genes (Young and Kelly 1996a), one of which has been previously described (*Co-5*), the two other genes need to be characterized. Since no discriminating race(s) of the pathogen is available to distinguish effectively between *Co-4*^{2} and *Co-7* resistance genes, it is impossible to know which gene or gene combination is being transmitted to the progeny. When combined in a single genotype, those unknown resistance genes, that cannot be identified by traditional pathological techniques, can be distinguished from each other using a marker tightly linked to one of the resistance genes. The use of markers to dissect the genetic basis of complex traits in other crops has been described (marker assisted dissection, Allard 1996).

In this study the use of tightly linked RAPD and SCAR markers to assist in the identification of independently inherited resistance genes for which no discriminating races of the pathogen are available is discussed. To demonstrate the application of marker assisted dissection, the anthracnose resistance genes present in the bean cultivar G 2333 were studied. RAPD and SCAR markers linked to the *Co-4* gene were identified and used to isolate the third resistance gene present in G 2333.

MATERIALS AND METHODS

Genetic material. The study of resistance to anthracnose in G 2333 was previously investigated using two breeding lines, SEL 1360 and SEL 1308, derived through backcrossing with G 2333. The cultivar G 2333 is highly photoperiod sensitive, which makes it difficult to work with in temperate regions. Both lines were resistant to a number of races of *C. lindemuthianum*, and each line was known to carry different resistance gene(s) since they displayed different phenotypic reactions after inoculation with a wide array of races of the pathogen (Young and Kelly 1996a). SEL 1360 possesses a single dominant resistance gene characterized as *Co-5*. Resistance in SEL 1308, however, was concluded to be similar to G 2333 parent, since no race was found to discriminate between the resistance genes present (Young and Kelly 1996a). Crosses between SEL 1308 and the cultivars Catrachita (*Co-6* gene; Beebe and Pastor-Corrales 1991) and Black Magic (susceptible; Kelly et al. 1987) were made and these two segregating populations were used to characterize the genetic resistance in SEL 1308 (Young et al. 1998). The Catrachita/SEL 1308 and Black Magic/SEL 1308 populations were composed of 72 and 99 F_2 individual plants, respectively, and the $F_{2:3}$ progeny of each individual F_2 plant were tested to discriminate homozygous from heterozygous resistant genotypes. Inheritance and allelism tests have shown that SEL 1308 possesses a single dominant gene conditioning resistance to anthracnose, the *Co-4*^{2} gene, which is allelic to the *Co-4* gene present in the differential cultivar TO (Young et al. 1998). SEL 1360 (*Co-5* gene) was crossed with the original parent G 2333 to generate a population where the unknown genes would be segregating and the *Co-5* gene would be fixed.

RAPD and SCAR analyses. Individual F_2 plants from Catrachita/SEL 1308 and Black Magic/SEL 1308 populations were used as two independent mapping populations to test for putative linkages between RAPD markers and the resistance gene in SEL 1308. Bulked segregant analysis (Michelmore et al. 1991) was used to form two independent and contrasting DNA bulks composed of equal volumes of fluorometrically standardized DNA from six F_2 homozygous resistant and six F_2 homozygous susceptible plants derived from both mapping populations. The PCR procedure was similar to that described by Young and Kelly (1996b) using random primers (Operon Technologies, Inc., Alameda, CA) and Stoffel DNA polymerase (Perkin Elmer). The thermal profile used consisted of 3 cycles of 1min at 94° C, 1min at 35° C, 2min at 72° C; 34 cycles of 10s at 94° C, 20s at 40° C, 2min at 72° C, followed by 1 cycle of 5min at 72° C. The RAPD amplification products linked to the *Co-4*[2] gene were purified using the QIAquick gel extraction kit (Qiagen Inc., Chatsworth CA) and cloned by means of the TA cloning system (Invitrogen Corporation, San Diego, CA). Cloned amplification products were sequenced following the procedure described by Melotto et al. (1996, Appendix A1). SCAR markers were generated from the RAPD markers and tested in both mapping populations. Amplification of the DNA fragment by the SCAR primers was carried out as described by Melotto et al. (1996). The PCR procedure consisted of 34 cycles of 10s at 94° C, 2 min 40 sec at 72° C, followed by 1 cycle of 5min at 72° C. The presence or absence of DNA amplification was confirmed in the parental genotypes and in selected samples from the two populations using Taq DNA polymerase (Gibco, BRL). In addition, the markers were used for purposes of marker assisted selection to identify F_2 individuals, which did not possess the marker and to screen other differential and commercial cultivars.

Linkage analysis. Simple inheritance of the disease phenotype and the putative linked RAPD and SCAR markers was confirmed using Chi-square tests. Linkage analysis was performed using the program Linkage-1 (Suiter et al. 1983). Linkage estimates between loci are expressed in centimorgans (cM), as calculated using Kosambi's function by the Linkage-1 computer program.

RESULTS

A total of 260 decamer primers were used to screen for RAPD markers linked to the *Co-4*^{2} gene and two linked RAPD markers were identified. One marker designated OAS13$_{950}$ (generated by a 5'-CACGGACCGA-3' decamer; Figure 2.1A) co-segregated with the resistance gene in 143 individuals in both populations and no recombinants were observed (Table 2.1). This marker was present in SEL 1308, G 2333 and in the differential cultivars, Widusa, PI 207262 and TO which carries the *Co-4* gene. The marker was absent from all other differential cultivars including SEL 1360 and those with characterized resistance genes. In a survey of 24 commercial bean cultivars representing the two gene pools of *P. vulgaris*, the OAS13$_{950}$ RAPD marker was present in four related navy bean cultivars, Monroe, Sanilac, Seafarer and Seaforth. Similar results were observed using Taq DNA polymerase (Figure 2.1B).

The two specific 24-mer SCAR primers, synthesized based on the DNA fragment sequence amplified by the OAS13$_{950}$ RAPD marker, are shown in Table 2.2. The SCAR marker SAS13 amplified a single DNA fragment of the expected size (950 bp) and appeared as a single polymorphic band in agarose gels (Figure 2.1C). In addition, SAS13 followed the same co-segregation with the *Co-4*^{2} allele as the OAS13$_{950}$ RAPD marker in both F_2 populations, supporting the tight linkage between the marker and the resistance gene.

Table 2.1 Chi-square analysis for the $OAS13_{950}$ RAPD marker locus segregating in two F_2 populations, two-point chi-square analysis, and linkage estimates for the marker locus and the *Co-4²* resistant allele.

Population	Locus tested	Expected ratio	Observed frequency	X^2	P[a]	cM[b] (r ± SE)
Catrachita/SEL 1308	$OAS13_{950}$	3:1	47:19	0.32	0.57	
Black Magic/SEL 1308	$OAS13_{950}$	3:1	70:25	0.03	0.86	
Catrachita/SEL 1308	$OAS13_{950}$/*Co-4²*	3:6:3:1:2:1	16:29:0:0:0:17	62.0	0.0	0.0 ± n.d.
Black Magic/SEL 1308	$OAS13_{950}$/*Co-4²*	3:6:3:1:2:1	12:45:0:0:0:24	81.0	0.0	0.0 ± n.d.
Across populations	$OAS13_{950}$/*Co-4²*	3:6:3:1:2:1	28:74:0:0:0:41	143.0	0.0	0.0 ± n.d.

[a] P = probability estimated value.

[b] Linkage analysis based on 1:2:1 genotypic segregation ratio for the *Co-4²* locus and 3:1 ratio for the $OAS13_{950}$ RAPD marker.

Table 2.2 SCAR primer sequences derived from the RAPD marker $OAS13_{950}$ linked to the *Co-4²* allele in common bean.

SCAR marker	Primer	Sequence[a]
SAS13	SAS13.24XP	5'-CACGGACCGAATAAGCCACCAACA-3'
	SAS13.24RP	5'-CACGGACCGAGGATACAGTGAAAG-3'

[a]Underlined sequences are the original $OAS13_{950}$ RAPD marker.

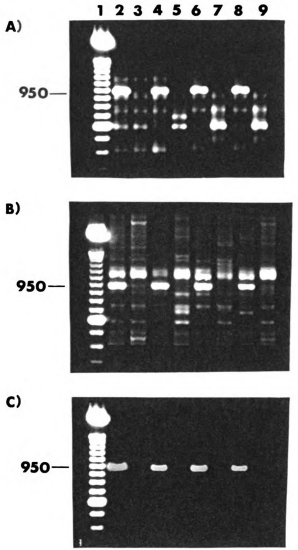

Figure 2.1 DNA amplification of eight bean cultivars using **A)** the $OAS13_{950}$ RAPD marker with Stoffel DNA polymerase, **B)** the $OAS13_{950}$ RAPD marker with Taq DNA polymerase, **C)** the SAS13 SCAR marker. Lanes (1) 100 bp DNA ladder, (2) Resistant DNA bulk (*Co-4²/Co-4²*), (3) Susceptible DNA bulk (*co-4²/co-4²*), (4) SEL 1308 (*Co-4²/Co-4²*), (5) SEL 1360 (*co-4²/co-4²,Co-5/Co-5*), (6) G 2333 (*Co-4²/Co-4²,Co-5/Co-5*), (7) Catrachita (*Co-6/Co-6*), (8) TO (*Co-4/Co-4*), (9) Black Magic (*co-4²/co-4²*).

The $OAS13_{950}$ and SAS13 markers were used to identify F_2 individuals, which did not possess the *Co-4²* allele in the SEL 1360/G 2333 population. A progeny test of F_3 families (derived from F_2 individuals without the $OAS13_{950}$ marker) was performed using race 521 to discriminate homozygous resistant *co-4²co-4²/Co-7Co-7* genotypes from the heterozygous *co-4²co-4²/Co-7co-7* individuals. Six F_2 individuals, in the SEL 1360/G 2333 population, were first identified as susceptible to race 521, thus are recessive for both genes (Table 2.1). Only 55 individuals from the remaining 86 F_2 individuals were harvested due to problems with photoperiod sensitivity in the cross. Among these, 14 individuals lacking the $OAS13_{950}$ marker were identified. The F_3 progeny (consisting mainly of 15 individuals) of these 14 F_2 individuals were screened with race 521 and 3 homozygous resistant F_3 families (*co-4²co-4²/Co-7Co-7*) consisting of 15 individuals were identified (Table A2.1). One of these F_3 families, named SEL 111 was chosen for crossing with the susceptible cultivar Black Magic and members of the anthracnose differential series. The derived populations will be used in allelism tests with other known resistance genes to fully characterize the *Co-7* gene (Appendix A2).

DISCUSSION

The characterization of the genetic resistance of common bean to *C. lindemuthianum* in G 2333 is of major importance to breeders because of the broad based resistance exhibited by this cultivar (Pastor-Corrales et al. 1994). The combination of resistance genes present in G 2333 would be of great utility for long term resistance if bred into commercial bean cultivars. In this study, we confirm that G 2333 carries three independently inherited resistance genes, one of which is a second resistance allele, *Co-4*^{2}, at the *Co-4* locus. The finding of different alleles at the same locus conferring differential resistance to races of *C. lindemuthianum* is not unique (Fouilloux 1979). The second allele at the *Co-4* locus offers improved potential in breeding for anthracnose resistance since it conditions resistance to a broader array of pathogenic races, similar to the second allele at the *bc-2* locus which conditions resistance to bean common mosaic virus (Drijfhout 1991).

The current study supports the importance of Mexican bean germplasm as a valuable source of resistance to anthracnose. Five of the 12 differential cultivars either come directly from Mexico or were derived through crossing with Mexican germplasm. Two Mexican landrace cultivars, PI 207262 named Tlalnepantla 64 and G 2333 named Colorado de Teopisca (Pastor-Corrales et al. 1994), are used directly as differentials. Fouilloux (1979) identified the three *Mexique* genes (*Co-3, Co-4, Co-5*) in Mexican germplasm and transferred them into the differential cultivars, Mexico 222, TO, and TU, respectively. G 2333 carries additional resistance genes (Young and Kelly 1996a), whereas the resistance gene(s) in PI 207262 has not been characterized fully (Gonçalves-Vidigal et al. 1997). Although the *Co-5* gene in G 2333 is the same *Mexique III* gene in TU, the second gene in G 2333 is unique as

it is an allele of the *Co-4 (Mexique II)* gene with a broader resistance pattern. The third gene, *Co-7*, appears to offer equal potential in breeding for resistance since, in combination with the *Co-4*^{2} allele, it has not been overcome by any existing race of the pathogen (Balardin et al. 1997, Pastor-Corrales et al. 1994). Bean breeders interested in controlling anthracnose may wish to utilize Mexican germplasm as a source of new and useful Middle American genes for resistance to anthracnose. This is analogous to the research on breeding for rust resistance in common bean where germplasm from Guatemala appears to offer the best potential as a source of resistance to that variable pathogen (Stavely 1990).

RAPD and SCAR markers linked to anthracnose resistance genes in common bean have been reported previously (Adam-Blondon et al. 1994, Young and Kelly 1996b, 1997). However, the finding of the $OAS13_{950}$ marker in the three differential cultivars, Widusa, PI 207262 and TO was unexpected. Finding the markers only in G 2333 was anticipated so their presence in the differential cultivar TO (*Co-4* gene) raises some question about the linkage with the *Co-4*^{2} allele. Since no recombinants were observed between the $OAS13_{950}$ marker and the *Co-4*^{2} allele in two populations, a tight linkage is assumed. Similar tight linkages have been observed in common bean for the rust resistance *Ur-4* gene and the $OA14_{1100}$ marker (Miklas et al. 1993). One major difference between these studies is that the $OAS13_{950}$ marker was detected in two populations (143 individuals) with similar genetic backgrounds, i.e., Middle American gene in a Middle American background, whereas Miklas et al. (1993) found no recombinants (70 individuals) between the $OA14_{1100}$ marker and the Andean *Ur-4* gene. Recombination was restricted in the latter population because the Andean *Ur-4* gene had been backcrossed into genetically distant Middle American germplasm. Young and Kelly (1996b) observed lower recombination values between RAPD markers and the Middle

American *Co-2* gene expressed in an Andean background than in a Middle American background. The presence of the $OAS13_{950}$ marker in two cultivars, TO and SEL 1308, which carry alleles at the *Co-4* locus, is not a major limitation in breeding for resistance. The markers can be used to indirectly select either allele at the *Co-4* locus depending on the germplasm available to the breeder. The presence of the markers in Widusa and PI 207262 suggests that they possess a different allele at the *Co-4* locus in addition to other resistance gene(s). Since neither Widusa nor PI 207262 possess greater levels of resistance than TO, they cannot carry either characterized *Co-4* allele. Allelism tests among Widusa, PI 207262, TO, and SEL 1308 are planned since the resistance gene(s) in Widusa and PI 207262 has not been characterized fully.

The presence of the markers in the navy bean cultivars, Monroe, Sanilac, Seafarer and Seaforth has no obvious explanation. Other related navy bean cultivars such as Michelite, Mayflower, C-20, Bunsi, Kentwood, Fleetwood, Harofleet, Harokent, OAC-Rico were screened, but the marker was absent. The marker was absent from all other market classes of dry bean and snap bean surveyed. Since all bean genotypes with the $OAS13_{950}$ RAPD and SCAR markers possess different genes for resistance to anthracnose, possibly a cluster of anthracnose resistance genes exists at or near the *Co-4* locus. The existence of clusters of resistance genes has been demonstrated in *Lactuca* and *Lycopersicon* spp (Maisonneuve et al. 1994, Salmeron et al. 1996). Although the *Co-1* gene in Sanilac and Seafarer, and the *Co-2* gene in Seaforth are independent of the *Co-4* locus, all these cultivars carry additional resistance to anthracnose races *beta* and *gamma* (Cardenas et al. 1964). The resistance in Monroe is unknown, but it may carry the same resistance to races *beta* and *gamma* as its parent, Michelite. In common bean, Adam-Blondon et al. (1994) mapped nine

genes of known function, the majority of which represent gene families such as the 3-10 genes for phaseolin storage protein, six genes for chalcone synthase, and four for phenylalanine ammonia lyase. In all cases only one member of the gene family was mapped (Adam-Blondon et al. 1994). The presence of clustered genes for phaseolin supports the theory that specific genes for anthracnose resistance may exhibit a similar clustering pattern based on the presence of the marker in genotypes with different resistance genes. Additional evidence for the presence of complex loci of linked resistance genes in common bean was demonstrated for genes controlling resistance to bean rust at the *Ur-5* locus (Stavely 1984, Appendix A3).

Although the broad-based anthracnose resistance of G 2333 has been widely tested and confirmed by different research groups (Balardin et al. 1997, Pastor-Corrales et al. 1994, Schwartz et al. 1982), the identification of individual genes is needed to evaluate their potential in breeding for resistance. In the absence of a race of the pathogen virulent on G 2333, the use of tightly linked markers, as suggested by Maisonneuve et al. (1994), to identify and combine different genes was evaluated. In the F_2 population (SEL 1360/G 2333) where the *Co-5* gene is fixed and the *Co-4²* and *Co-7* genes are segregating independently, six of the nine possible genotypic combinations will carry at least one dominant *Co-4²* allele. The other three genotypes would be homozygous for the recessive *co-4²* allele, one of which is the double recessive *co-4²co-4²/co-7co-7*, identified by its susceptible reaction to race 521 which is also virulent to the *Co-5* gene. All three genotypes were identified by selecting F_2 individuals which do not possess the $OAS13_{950}$ RAPD and SAS13 SCAR markers linked in coupling with the *Co-4²* allele. By eliminating the double recessive individuals based on inoculation, the other two genotypes were identified. These genotypes possess the recessive

co-4² allele and differ at the *Co-7* locus for homo- or heterozygosity. The homozygous individuals were identified as pure breeding resistant F_3 families after inoculation with race 521. In addition, the $OAS13_{950}$ RAPD and SCAR markers were used to construct the resistant and susceptible DNA bulks for screening to detect markers linked to the *Co-7* gene. Individuals included in the susceptible bulk were detected in the SEL 1360/G 2333 population after inoculation with race 521 of the pathogen. We confirmed that all members of the susceptible bulk did not have the $OAS13_{950}$ marker as expected. Since no race is available to distinguish the *Co-4²* and *Co-7* genes, only those resistant individuals which do not possess the $OAS13_{950}$ markers linked to the *Co-4²* gene will be included as members of the resistant bulk. This ensures that the resistance to race 521 can only be derived from the *Co-7* gene. Screening of the bulks with random decamer primers will be conducted to find a coupling RAPD marker linked to the new resistance gene, since the resistant F_2 heterozygotes were not distinguished from the F_2 homozygotes when the resistant bulk was formed. Once detected, the marker can be used to ensure that the *Co-7* resistance gene is incorporated into different genetic backgrounds, independent of other anthracnose resistance genes.

Finally, the use of this information in developing a better differential series for the classification of new races of anthracnose is proposed. Despite its broad resistance, G 2333 is not the ideal differential cultivar since it carries three different resistance genes. One suggestion would be to replace G 2333 with SEL 1308 shown to carry only the single *Co-4²* allele and consider including upon identification, a genotype known to carry only the *Co-7* gene. The development of lines in which the *Co-7* gene is present alone becomes a priority. The *Co-7* gene represents a unique and important source of resistance to anthracnose and

should be invaluable, when fixed alone in a host genotype, to better classify potential new races of the anthracnose pathogen and be used in breeding durable anthracnose resistant germplasm.

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CHAPTER THREE

THE *COK-4* GENE IS PART OF A COMPLEX LOCUS CONDITIONING RESISTANCE TO ANTHRACNOSE IN COMMON BEAN, *PHASEOLUS VULGARIS*

ABSTRACT

Genetic mapping is a powerful approach to clone disease resistance genes. In this study, a tightly linked SCAR marker, SAS13 tested in 1018 F_2 individual plants, was used as a starting point for cloning gene sequences associated with the *Co-4* locus, which conditions resistance to the fungal pathogen *Colletotrichum lindemuthianum* in common bean. A contig developed from genomic clones flanking the marker region revealed an 1110 bp open reading frame, named *COK-4*. Two essential eukaryote promoter elements, TATA and CAAT boxes, and putative promoter sequences were found upstream of the *COK-4* gene. The predicted COK-4 protein contains a serine-threonine kinase domain with a highly hydrophobic membrane-spanning region. Specific primers designed to amplify the *COK-4* region, were used to clone and sequence *COK-4* homologs from different bean cultivars. Single nucleotide polymorphisms were found between the homologous sequences and were further confirmed with three restriction enzymes. Restriction patterns were polymorphic among three bean cultivars known to possess different alleles at the *Co-4* locus, Black Magic (*co-4*), TO (*Co-4*), and SEL 1308 (*Co-4*^{2}) as indicated by genetic analysis. Perfect co-segregation between restriction patterns and

disease phenotype was observed in 1350 F_3 individuals. More than one copy of the *COK-4* gene analog exists in the bean genome as demonstrated by Southern analysis. These results strongly suggest that the *COK-4* gene is a member of the complex *Co-4* locus conditioning resistance to anthracnose in bean. *COK-4*, a gene ortholog of the *Pto* in tomato, is the first disease resistance gene successfully cloned from legumes. Molecular cloning of resistance genes should facilitate studies on plant-pathogen interaction and ultimately facilitate genetic improvement of crop species.

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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). Seven independent dominant disease resistance genes (*Co-1* to *Co-7*) controlling anthracnose in bean have been described (Balardin et al. 1997). 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 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-4*^{2} 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-4*^{2}, *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-4*^{2} gene in SEL 1308 exhibits the broadest-based resistance in common bean (Balardin and Kelly 1998).

The *Co-4²* gene is a valuable candidate gene for molecular cloning due to its broad resistance and availability of a tightly linked marker (Young et al. 1998). To better understand the mechanisms of disease resistance several disease-resistance genes have been cloned from different plant species. Sequence analysis indicated that these genes encode structurally similar proteins with conserved function across plant species (Bent 1996). The opportunity exists, therefore, to identify resistance gene candidates from diverse plant taxa. In common bean and soybean, resistance genes analogs (RGAs) were identified using primers specific to conserved regions of known resistance genes and mapped close to known disease resistance locus (Kanazin et al. 1996, Yu et al. 1996, Geffroy et al. 1998, Rivkin et al. 1999). Mapping of RGAs has been the only 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 known resistance locus limiting their value in chromosome walking to the gene. Additional approaches, such as map-based cloning are still needed to isolate resistance gene candidates in crop species.

In the present study the fine mapping of the *Co-4²* locus using a tightly linked molecular marker is described. A previously described SCAR marker (Young et al. 1998) was used as the starting point for the cloning of gene sequences associated with the *Co-4²* anthracnose resistance gene. Cloned sequences were compared to known resistance gene sequences and the function of candidate resistance genes in common bean is discussed.

MATERIALS AND METHODS

Genetic analysis of the segregating population. The bean breeding line SEL 1308 obtained from CIAT was used as the source of the *Co-4²* gene. This line was derived from a backcross between the anthracnose susceptible cultivar Talamanca and the recurrent parent Colorado de Teopisca (accession number G2333). SEL 1308 was crossed to Black Magic, a susceptible black bean cultivar. 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. Race 73 (ATCC 96512) of *C. lindemuthianum* was chosen to confirm the dominant inheritance of the *Co-4²* gene in SEL 1308. Black Magic, the susceptible parent of the mapping population, dies in five days after inoculation. Inoculum preparation, inoculation methods, and disease characterization of the segregating population were conducted as described by Young and Kelly (1996). Individual F_2 plants from Black Magic/SEL 1308 population were screened with the SCAR marker SAS13 previously found to be linked to the *Co-4²* gene. Procedures for SCAR analysis are described elsewhere (Melotto et al. 1996). Additional RAPD markers flanking the *Co-4²* gene were sought by using bulked segregant analysis (Michelmore et al. 1991). Inheritance of 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 distance between marker and resistance gene expressed in centiMorgan (cM) was calculated using Kosambi's function in the Linkage-1 program.

Southern analysis. Genomic DNA from bean cultivars was digested with *Eco*R I according to the manufacturer (Boehringer Mannheim, Indianapolis, IN). Electrophoresis and blotting were conducted using standard techniques (Sambrook et al. 1989). The SAS13 marker was labeled using the Gibco RadPrime kit (Life Technologies, Inc., Rockville, MD) and used as a probe for hybridization. Stringency washes were performed in 2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS solutions. Both washes were conducted twice for 30 minutes 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, CA). DNA from SEL 1308 was purified using phenol and chloroform, and digested with five restriction enzyme, *Dra* I, *Eco*R V, *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 50ul solution containing 1x Advantage Genomic Polymerase Mix (Clontech Laboratories, Inc., Palo Alto, CA), 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 7 cycles of 2 seconds at 94°C, 4 minutes at 70°C, followed by 32 cycles of 2 seconds at 94°C, 4 minutes at 65°C and an extension cycle of 7 minutes at 65°C. Long distance PCR (LD-PCR) amplification products were cloned using the TOPO™ TA Cloning kit (Invitrogen Corp., San Diego, CA). 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 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, MI). 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 was deduced from the consensus DNA sequence and 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 primers, 1x enzyme buffer containing $MgSO_4$ and 1U of *Pfu* DNA polymerase (Promega, Madison, WI). PCR reactions were placed in a 9600 Thermocycler (Perkin Elmer Applied Biosystems) and the PCR file consisted of 34 cycles of 20 seconds at 95°C, 30 seconds at 55°C, and 4 minutes at 72°C, followed by an extension cycle of 7 minutes at 72°C. The amplification product was used in a digestion reaction containing 1x enzyme buffer, 10U of restriction enzyme and 10µl of PCR reaction. Digestion of DNA fragment was carried out for four hours at 37°C. Restriction patterns were observed on 0.8% ethidium bromide-stained agarose gel.

RESULTS

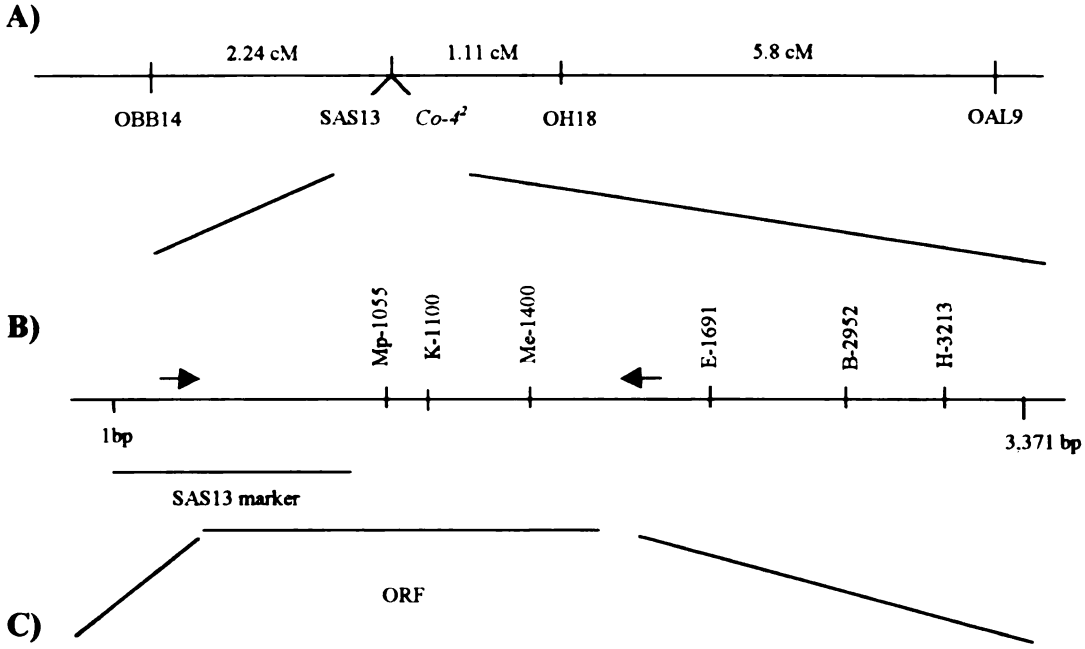
The original SAS13 SCAR marker, which amplified a single 950-bp fragment in the resistant parent, co-segregated with the *Co-4*^{2} resistance gene in the segregating population of 1018 F_2 individuals (Figure 3.1A). Four recombinant individuals were detected and the distance between the marker and the *Co-4*^{2} locus was estimated at 0.39 cM (Table 3.1). Three susceptible individuals possessing the SAS13 fragment and one resistant line lacking the fragment were observed. The 950-bp DNA fragment generated by the SAS13 SCAR marker (Young et al. 1998) was sequenced and analyzed for similarities to 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; Martin et al. 1993) and *Fen* gene (gi|1098334; 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. Four overlapping clones extending the original SAS13 950-bp fragment were obtained and the full length of the contig included 3,371 bp (Figure 3.1B). 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).

Table 3.1 Chi-square analysis of the SAS13 marker locus segregating in Black Magic/SEL 1308 F_2 population, two-point chi-square and linkage estimates for the marker locus and the *Co-4²* resistance allele.

Locus	Expected Ratio	Observed Frequency	*P**	cM ($r \pm$ SE)†
SAS13	3:1	753:265	0.45	
Co-4² co-4²	3:1	751:267	0.37	
SAS13 / *Co-4²*	9:3:3:1	750:1:3:264	0.0	0.39 ± 3.14

**P* = probability estimated value

† Linkage analysis based on 1:2:1 genotypic segregation ratio of the *Co-4²* and OBB14 marker loci and 3:1 ratio for OAL9, SAS13, and OH18 markers.



MFLNCVGMCCSKPTTNTTSSQRQFPTLIEELCHQFSLTDLRKATNNFDQKRV

IGSGLFSEVYKGCLQHDGASDYTVAIK

RFDYQGWAAFNKEIELLCQLRHPRCVSLIGFCNHE

NEKILVYEYMSNGSLDKHLQEGQLSWKKRLEICIGVARGLHFLHTGAKRSI

FHCILGPGTVLLDDQMEPKLAGFDASEQGSRFMSKQ

KQINVIVFWVIFVLLYELTHCHDF

LWIKLSLLFVIGCRGYTATDYLMDGIITAK

WDVFSFGFLLLEVVCRRMFYLIT

LTKKECLENPVEERIDPIIKGKIAPDCWQVFVDMMVSCLKYEPDERPTIGEVEVQLEHALSMQE

QSDITNSNSEYTLLSKTIISLGVKKCK•

Figure 3.1 Diagram of the genomic region containing the *Co-4²* locus. **(A)** Linkage map showing position of molecular markers. **(B)** Contig developed from overlapping genomic clones. Arrows indicate *COK-4* specific primers and restriction sites are letter coded, B=*BamH*I, E=*EcoR*I, H=*Hind*III, K=*Kpn*I, Me=*Mse*I, Mp=*Msp*I. **(C)** Detail of the COK-4 amino acid sequence showing color coded putative domains: red = N-myristoylation sites, green = N-glycosylation sites, pink = cAMP and cGMP-dependent protein kinase phosphorylation site, blue = protein kinase ATP-binding region signature, underlined amino acids = primary transmembrane region, waved underlined amino acids = secondary transmembrane region, • = stop codon. The original SAS13 marker site included COK-4 amino acids 1 through 173.

Sequence analysis of the contig revealed an open reading frame (ORF) of 1110 bp, which was named *COK-4*. 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 by the *Pto* gene from *Lycopersicon pimpinellifolium* and *L. esculentum* (38% identity, 53% similarity and 15% gap; Martin et al. 1993), TMK protein from rice (29%, 45%, and 16%; van der Knaap et al. 1996), extracellular S-domain from *Brassica oleracea* (30%, 45%, 15%; PID:g2598271) (Figure 3.2), S-domain receptor-like protein kinase from *Z. mays* (33%, 49%, 14%; PID:g3445397), and leucine-rich repeat (LRR) transmembrane protein kinase 2 from *Z. mays* (28%, 45%, 19%; Li and Wurtzel 1998). The protein encoded by the *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) (Figure 3.1C).

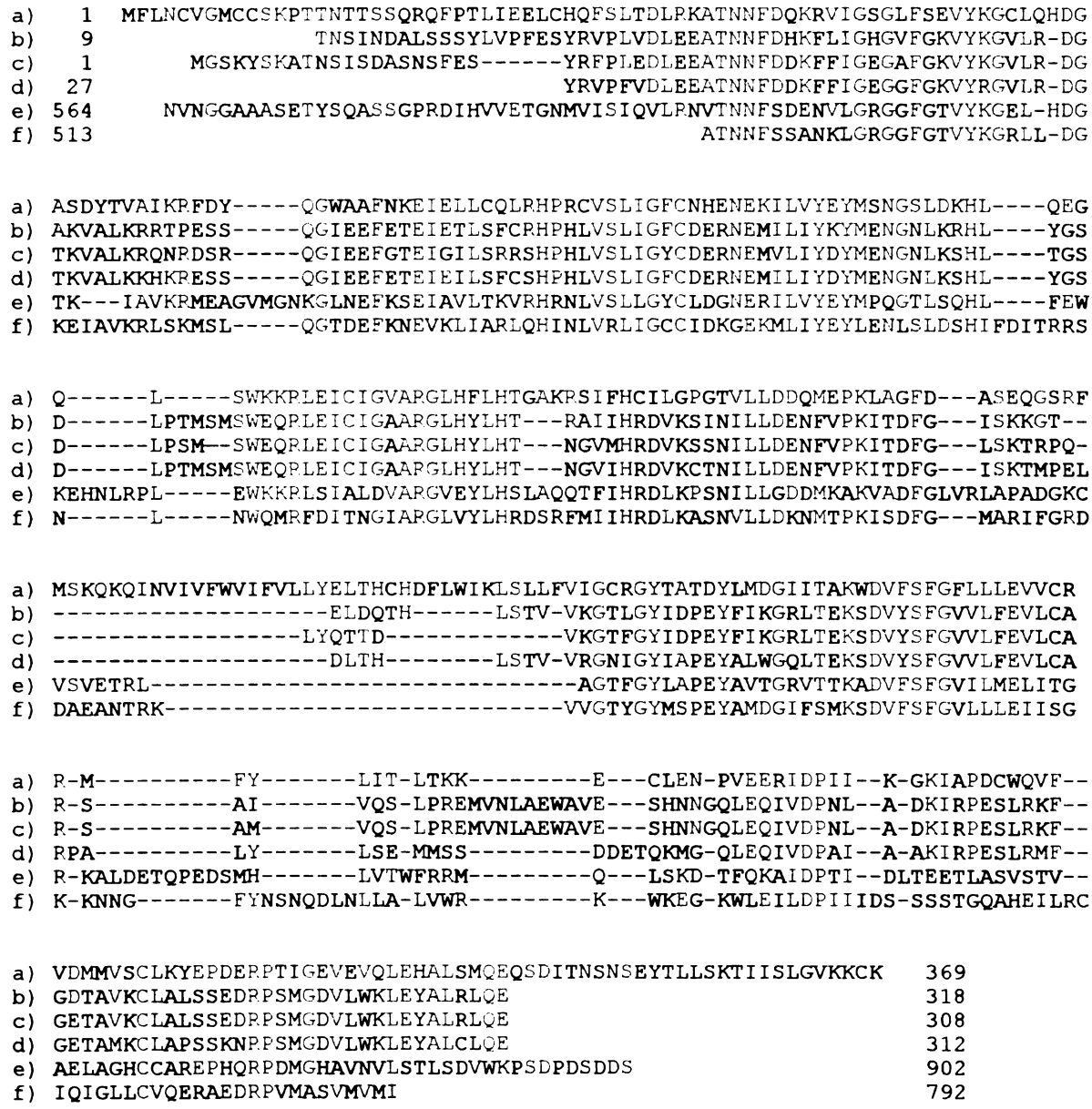


Figure 3.2 Alignment from regions of similarity between the COK-4 protein sequence and reported protein sequences. Amino acid identity is indicated in red and amino acid similarity is indicated in blue. Numbers in the sequence indicate the first and last amino acids aligned. a) COK-4 protein (GenBank data base accession no. AF153441); b) disease resistance protein kinase Pto gi|430992; c) serine/threonine protein kinase Pto (*Lycopersicon esculentum*) gi|1809257; d) putative serine/threonine protein kinase, *Fen* gene (*L. esculentum*) gi|557882; e) TMK (*Oryza sativa*) gnl|PID|e267533; f) extracellular S domain of *Brassica oleracea* gnl|PID|e1172841.

Two specific primers were designed to amplify the *COK-4* gene (Table 3.2). 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 (Figure 3.3A). Both parents of the mapping population and all individual bean cultivars tested possessed the *COK-4* gene. To search for nucleotide polymorphisms between the resistant and susceptible parents, the 1150 bp DNA fragments were cloned and sequenced. Alignment of these two sequences revealed a nucleotide identity of 98% between resistant and susceptible parents (Figure 3.4). The predicted amino acid sequence from the susceptible parent was interrupted by several stop codons. Based on the sequence data a restriction map at that region was predicted (Figure 3.1B) 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 nucleotide polymorphism between the parental genotypes and 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 (Figure 3.3B-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. Co-segregation of *COK-4* restriction patterns with disease phenotype was confirmed in 75 F_2 individual plants. All susceptible and heterozygous plants (genotyped as F_3 families) had more than one restriction site similar to Black Magic, whereas all homozygous dominant individuals had only one restriction site similar to SEL 1308. Restriction analysis of the bean cultivar TO, known to possess a different resistance allele at the *Co-4* locus revealed a third restriction pattern (Figure 3.3B-D). The *COK-4* homolog found in TO was sequenced and aligned with SEL 1308 and Black Magic

homologs (Figure 3.4). 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. TO and Black Magic nucleotide sequences were 94% identical.

To determine the copy number of the *COK-4* gene in different bean cultivars, *Eco*R I-restricted DNA was probed with the SAS13 marker (Figure 3.5). The resistant cultivars SEL 1308, G2333, and Seafarer possessed two major homologous DNA sequences of 1.5 and 9 kb in size, whereas the susceptible cultivars SEL 1360 and Cardinal possessed multiple homologous sequences of various sizes. Again, TO possessed a unique RFLP pattern with only one 9-kb DNA fragment.

Table 3.2 Sequence of the specific primer set designed to amplify the *COK-4* gene in the vicinity of the *Co-4*^{2} locus.

Primer	Nucleotide position	Sequence (5'-3')
1	405-426	GTA TGG TAA GTG ACA AGT GAG A
2	1578-1556	ACC TGG TCA CTT ACA TTT CTT CA

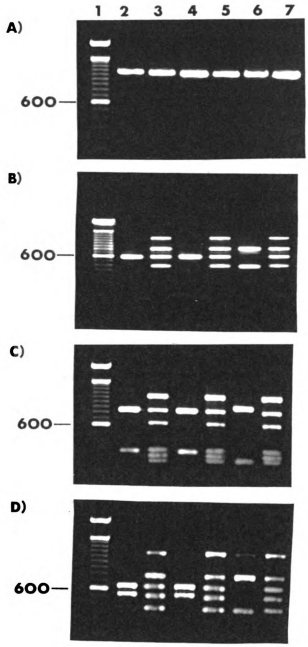


Figure 3.3 Restriction analysis of the *COK-4* amplified in several genotypes. (**A**) Undigested; (**B**) *Kpn* I; (**C**) *Mse* I; (**D**) *Msp* I. Lane (1) 100-bp DNA ladder, (2) SEL 1308 (*Co-4²/Co-4²*), (3) Heterozygous resistant F_2 plant (*Co-4²/co-4²*), (4) Homozygous resistant F_2 plant (*Co-4²/Co-4²*), (5) Homozygous susceptible F_2 plant (*co-4²/co-4²*), (6) TO (*Co-4/Co-4*), (7) Black Magic (*co-4²/co-4²*).

```
A) 1    atgtttctgaattgtgtgggcatgtgttgttcgaagcccacaacaaatacaacttcatct
B)      atgtttctgaattgtgtgggcatgtgttgttcgaagcccacaacaaatacaacttcatct
C)      atgtttctgaattgtgtgggcatgtgttgtacgaagcccacaacaaatacaacttcatct

A) 61   cagagacagtttccaacgttgatagaagagctgtgccatcaattttctctcaccgatctt
B)      cagagacagtttccaacgttgatagaagagctgtgccatcaattttctctcaccgatctt
C)      cagagacagtttccaacgttgatagaagagctgtgccatcaattttctctctccgatctt

A) 121  aggaaagccaccaataactttgatcagaagagagtaataggaagtggattatttagtgaa
B)      aggaaagccaccaataactttgatcagaagagagtaataggaagtggattatttagtgaa
C)      aggaaagccatcaataactttgatcagaagagagtaataggaagtggatttttaggaa

A) 181  gtatacaaagggtgtctgcagcacgatggtgcttctgattacacggtcgcaataaagcga
B)      gtatacaaagggtgtctgcagcatgatggtgcttctgattacacggtcgcaataaagcga
C)      gtatattaagggtgtctgcagcatgatggtgcttctgattacacggtcgcaataaagcga

A) 241  tttgattatcaaggatgggcagcgttcaacaaggaaatcgaattgctatgccagcttcgt
B)      tttgattatcaaggatgggcagcgttcaacaaggaaatcgaattgctatgccagcttcgt
C)      tttgattatcaaggatgggaagcgttcaacaaggaaatcgaattgctatgccagcttcgt

A) 301  caccctagatgtgtttctcttataggattctgcaaccacgaaaatgagaagattcttgta
B)      caccctagatgtgtttctcttataggattcagcaaccacgaaaatgagaagattcttgta
C)      caccctagatgtgtttctcttataggattctgcaaccaccaaaatgagaagattcttgta

A) 361  tacgagtacatgtccaatggatctctagataaacacctacaagaaggtcaactatcatgg
B)      tacgagtacatgtccaatggatctctagataaacacctacaagaaggtcaactatcatgg
C)      tacgagtacatgtccaatggatctctagataaacacctacaagatggtgaactatcatgg

A) 421  aagaagaggctggagatatgcataggagtagcacgtggactacacttccttcacaccgga
B)      aagaagaggctagagatatgcataggagtagcacgtggactacactaccttcacaccgga
C)      aagaagaggctagagatctgcataggagtagcacgtggactacactaccttcacactggt

A) 481  gccaagcgttccatctttcactgtatcctcggtcctggtaccgtccttttggatgaccag
B)      gccaagcgttccatctttcactgtatcctcggtcctggtaccgtccttttggatgaccag
C)      gccaagcgttccatctttcactgtatcctcggtcctagtaccatccttttggatgaccaa

A) 541  atggagccaaaactcgctggtttcgatgctagcgagcagggatcacgttttatgtcaaag
B)      atggagccaaaactcgctggtttcggtgctagcgagcagggatcacgttttatgtcaaag
C)      atggagccaaaactcgctggtttcggtgttagcatgcagggatcacgttttatgtcaaag

A) 601  cagaagcaaatcaatgt-gatcgtgttttgggtaatttttgttttgttgtatgagctcac
B)      cagaagcaaatcaatgtagatcgtgttttgggtaatttttgttttttgtatgagctcac
C)      cagaagcaaatcaatgtagatcgtgttttgggtaatttttgttttgttgtatgagctcac
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```
A) 660  tcactgccatgatttttgtggatcaaactaagct--tactctttgttataggttgtagggg
B)      tcactgccatgatttttgtggatcaaactaagct--tactctttgttataggttgttgggg
C)      tcactgcaatgaattttttgtggatcaaactaagctaatactctttgttataggtacttttgg

A) 720  ctacacggctacggactatctcatggatggtatcatcacagctaaatgggatgttttctc
B)      ctacacggctacggactatctcatggatggtatcatcacagctaaatgggatgttttctc
C)      ctaccggctacggactatgtcatggatggtaccatcacagctaaatgggatgttttctc

A) 780  atttggtttccttctactagaagttgtgtgcaggaggatgttttatttaataactctgac
B)      atttggtttccttctactagaagttgtgtgcaggaggatgttttatttgataactctgac
C)      atttggtttccttctactagaagttgtgtgcaggaggatgttttatttgataactctgac

A) 840  taaaaaagaatgtctggagaatcctgttgaggagagaattgatccgattatcaaaggaaa
B)      taaaaaaaaatgtctggagaatcctgttgagtagagaattgatccgattatcaaagggaa
C)      taaaaaaaaatgtctggagaatcctgttgaggagagaattgatccgattatcaaagggaa

A) 900  gattgcaccagattgttggcaagtgtttgtagatatgatggtaagttgcttgaagtatga
B)      gattgcaccagattgttggcaagtgtttgtagatatgatggtaacttgcttgaagtataa
C)      gattgcaccagattgttggcaagtgtttgtagatatgatggtaacttgcttgaagtatga

A) 960  accagatgagagaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat
B)      accagatgagagaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat
C)      accagatgagcgaccaacaattggtgaagtggaggtgcaacttgagcatgctctatccat

A) 1020 gcaggaacaatctgatatcacaaactccaactctgagtataccttactctccaaaaccat
B)      gcaggaacaagctgatatcacaaactccaactctgagtatacttactgtccaaaaccat
C)      gcaggaacaagctgatatcacaaactccaactctgagtataccttactgtccaaaaccat

A) 1080 tatttcccttggagtgaagaaatgtaagtga 1110
B)      tatttccctgggagtgaagaaatgtaagtga
C)      tatttccc
```

Figure 3.4 DNA sequence showing single nucleotide polymorphisms (SNPs) in underlined red letters among three cultivars possessing different *COK-4* homologs, A) SEL 1308, B) Black Magic, and C) TO.

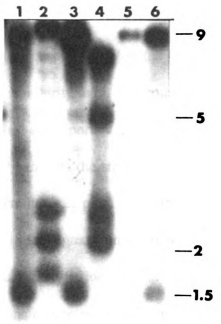

Figure 3.5 Southern analysis of *EcoR* I digested DNA of bean cultivars hybridized with the SAS13 marker. Lane (1) SEL 1308, (2) SEL 1360, (3) G2333, (4) Cardinal, (5) TO, (6) Seafarer. Molecular weight is indicated in kb.

DISCUSSION

Two lines of evidence strongly suggest that the *COK-4* gene, herein described, is a member of the complex *Co-4*^{2} locus conditioning resistance to anthracnose in common bean. First, genetic analysis indicated co-segregation of the SAS13 SCAR marker with the resistant phenotype in a population of 1018 F_2 individuals. Secondly, amino acid sequence analysis of the *COK-4* gene, which is located 462 bp downstream of the marker (Figure 3.2B), 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 RLKs. Receptor-like kinases contain an extracellular domain possibly functioning in ligand binding and a cytoplasmic domain responsible for signal transduction (Walker 1994). Unlike the Pto protein, 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 *Brassica oleracea* 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 occur at the membrane level. *Colletotrichum 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 maybe a host-specific elicitor located in the membrane and pathogen recognition may occur at the surfaces of the infection hyphae and host cell membrane.

Most of the disease resistance genes previously cloned confer resistance to bacterial diseases and are localized in the cytoplasm. Bacterial *Avr* gene products are known to be secreted in to the host cytoplasm through the type III secretory system (Bent 1996). However, little is known about the function of Avr proteins from fungal pathogens and only a few fungal Avr-generated signals have been described. Race-specific elicitors have been partially purified from two hemibiotrophic plant pathogens, *C. fulvum* and *C. lindemuthianum* (Lamb et al. 1989). One well-studied example of race-cultivar specificity is the *Cladosporium fulvum / Lycopersicon* pathosystem. Cf proteins possess extracellular domains, which supposedly recognize the corresponding Avr proteins (Jones et al. 1994, Dixon et al. 1996). Although Avr proteins of *C. lindemuthianum* have not been isolated, occurrence of race-cultivar specificity suggests the presence of 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 (Figure 3.4) and sequence analyses. Single nucleotide polymorphisms (SNPs) identified in the *COK-4* sequences of resistant and susceptible

bean lines and co-segregation of restriction patterns with disease phenotype, indicate that the *COK-4* gene is involved in anthracnose resistance. The four F_2 individuals found to be recombinants between the SAS13 marker and the *Co-4²* gene, however, possessed the *COK-4* allele corresponding to the phenotype of the plant (data not shown). 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). A SNP found in rice accounted for 80% of the variation in amylose content (Ayres et al. 1997). Small variation in gene sequences, therefore, can result in contrasting phenotypes.

Previous genetic studies indicated that the *Co-4* locus is a complex gene family (Young et al. 1998). Two resistance alleles have been described which mapped at the *Co-4* locus, one present in the bean cultivar TO and the other present in SEL 1308 as supported by allelism test and DNA sequence analysis. TO showed a unique restriction pattern and 44 nucleotide substitutions at the *COK-4* region compared to SEL 1308. 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-4²* locus. Bean cultivars appear to possess multiple copies of the *COK-4* gene based on Southern analysis. 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 must be linked to *COK-4* and may be a gene duplication based on the RFLP patterns. Another anthracnose resistance gene, *Co-2* has also been shown to be a complex multigene family (Geffroy et al. 1998). Sequence analysis of a linked marker revealed multiple copies of LRR sequences clustered near the *Co-2* gene. Resistance genes appears 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).

These findings indicate that tightly linked molecular markers may be used to identify disease resistance candidate genes. The SAS13 marker tagged the *Co-4* locus as it 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 as well as homologs present in the susceptible cultivars. By comparing these homologs, SNPs were identified and therefore, be more accurate than the SCAR marker in discriminating the plant phenotype. The four recombinant F_2 individuals from the mapping population between the marker and resistance locus, in fact, possessed the *COK-4* allele corresponding to the expected disease reaction confirmed by the restriction analysis. Most important, SNPs could be used to identify three different alleles at the *Co-4* locus. This work represents the first report of the successful cloning of a disease resistance gene in the plant family Leguminosae. The *COK-4* gene that conditions resistance to a fungal pathogen is a gene ortholog of the *Pto* resistance gene present in tomato.

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APPENDICES

APPENDIX A1

DEVELOPMENT OF A SCAR MARKER LINKED TO THE *I* GENE IN COMMON BEAN

ABSTRACT

Two 24-mer SCAR primers (SW13) were developed from a previously identified 10-mer RAPD primer ($OW13_{690}$) linked to the *I* gene, which conditions resistance to bean common mosaic virus (BCMV) in common bean. Linkage between SW13 and the *I* gene was tested in three F_2 populations segregating for both SW13 and the *I* gene: N84004/Michelite (1.0 ± 0.7 cM), Seafarer/UI-114 (1.3 ± 0.8 cM), and G91201/Alpine (5.0 ± 2.2 cM). SW13 proved to be more specific and reproducible than the $OW13_{690}$ RAPD marker. Using different heat stable DNA polymerases, SW13 amplified a single 690 bp fragment linked to the *I* gene which more consistently permitted the identification of resistant plants. In addition, the presence of the *I* gene was detected using SW13 in genotypes originating from different gene pools of *Phaseolus vulgaris* (L.), indicating a broad utility of this marker for bean breeding programs.

INTRODUCTION

Bean common mosaic virus (BCMV) is a widespread and economically important pathogen of common bean (*Phaseolus vulgaris* L.). The only effective way to prevent the occurrence of the disease caused by this pathogen is to develop genetically resistant cultivars (Kelly et al. 1995). Bean cultivars which carry the dominant *I* gene are resistant to all known races of BCMV. However, when bean plants are infected with strains of bean common mosaic necrosis virus (BCMNV), the presence of the *I* gene causes a lethal hypersensitive reaction in the plant (Haley et al. 1994a). Combining the *I* gene with other strain-specific recessive resistance genes (*bc-1*, *bc-2*^{2}, and *bc-3*) will protect the plant against BCMNV. However, using disease screening, it is not possible to detect the presence of the *I* gene in the *I/bc-3* gene combination (Morales and Kornegay 1996), because the recessive *bc-3* gene epistatically masks the action of the *I* gene (Kelly 1995). Hence, a reliable and specific molecular marker that can be used to indirectly select for the hypostatic resistant *I* gene is needed to breed for BCMNV resistance.

A single random amplified polymorphic DNA (RAPD) marker ($OW13_{690}$) has been found to be tightly linked in coupling with the *I* gene (ranging from 1.3 $\pm$ 0.8 to 5.0 $\pm$ 2.2 cM) in five segregating populations (Haley et al. 1994a). However, use of RAPD markers is restricted because they are very sensitive to variations in reaction conditions. A precise match between the RAPD primer and the template DNA depends on the choice of the heat-stable DNA polymerase and thermal cycler, the concentration of Mg^{++}, and the annealing temperature (Gu et al. 1995). Development of a specific repeatable protocol is

crucial for identifying robust polymorphisms and for obtaining consistent reproducibility of RAPD analysis (Kelly 1995).

Recently, a more reliable and specific PCR-based marker known as sequence characterized amplified region (SCAR) was developed. SCAR primers are longer than RAPD primers and a highly stringent annealing temperature can be employed which prevents mismatching in the priming site during DNA amplification (Paran and Michelmore 1993). Unlike RAPD, SCAR primers can amplify a single locus which appears as a single, easily-scored band in agarose gels.

In this research, the objectives were to (1) clone and sequence the DNA fragment amplified by RAPD marker $OW13_{690}$, (2) develop 24-mer SCAR primers with more specificity than $OW13_{690}$, (3) score the segregating bean populations developed by Haley et al. (1994a) for presence or absence of both *I* gene and marker, (4) identify the usefulness of these SCAR primers in amplifying the *I* locus across gene pools (Singh et al. 1991), and (5) test the robustness of the SCAR primers with different heat stable DNA polymerases.

MATERIALS AND METHODS

RAPD analysis. A RAPD marker linked to the *I* gene was identified using bulk segregant analysis (BSA, Michelmore et al. 1991) in near isogenic lines and linkage between this RAPD marker and the *I* locus was determined in five F_2 segregating bean populations. Development of these populations and their evaluation for resistance to BCMV have been previously described (Haley et al. 1994a).

Cloning and sequencing of the RAPD fragment. The RAPD amplification product linked to the *I* gene was purified and cloned using the PCR select spin column following procedures of the Primer PCR Cloner system (5 prime-3 prime, Inc.). Cloned amplification products were sequenced following the Sanger dideoxy-mediated chain terminator method (Sanger et al. 1977). This process involved two steps: a) preparation of DNA extension which consisted of 15 cycles of 20s at 94°C, 40s at 50°C, and 60s at 68°C followed by 15 cycles of 20s at 94°C, and 60s at 68°C, and b) separation of fragments on a polyacrylamide gel using the ABI 373A DNA sequencer. Double-stranded DNA sequencing was carried out using the -21M13 and M13 reverse fluorescently labeled primers.

SCAR analysis. Amplification reactions were carried out in 25µl solution containing 2 units of Stoffel DNA polymerase, 1X buffer, 5mM $MgCl_2$, 200µM of each dNTP, 25ng of DNA template and 25ng of SCAR primer mixture. The PCR procedure consisted of 34 cycles of 10s at 94°C, 40s at 67°C, and 2min at 72°C, followed by one

cycle of 5min at 72°C. Amplification products were detected by direct staining with 0.05 μg of ethidium bromide in microtiter plates and then observed in a 1.4% agarose gel.

Three out of five segregating populations developed by Haley et al. (1994a) (N84004/Michelite, Seafarer/UI-114, and G91201/Alpine) and several bean genotypes were screened using the SCAR primers. The presence or absence of the DNA amplification in selected samples of the three segregating populations was confirmed either with Ampli*taq* DNA polymerase (Perkin Elmer) or *Taq* DNA polymerase (BRL Gibco) using the same amplification protocol.

Linkage analysis. The recombination frequency between the SCAR marker and the *I* allele was calculated using the Linkage-1 software (Suiter et al. 1983).

RESULTS AND DISCUSSION

Two specific 24-mer SCAR primers were synthesized based on the DNA fragment sequence amplified by the RAPD marker. The 24-mer oligonucleotides contained the ten original base pairs of the RAPD marker plus the internal 14 base pairs (Table A1.1).

Table A1.1. SCAR primer sequences derived from the RAPD marker $OW13_{690}$ linked to the *I* gene in common bean.

SCAR marker	Primer	Sequence*
SW13	W13.7XP	5'-CACAGCGACATTAATTTTCCTTTC-3'
	W13.4RP	5'-CACAGCGACAGGAGGAGCTTATTA-3'

*underlined sequences are the original RAPD marker

The SCAR marker SW13 amplified DNA fragments of the expected size (690 bp) and appeared as a single polymorphic band in agarose gel (Figure A1.1). The polymorphic SCAR primers were then used to amplify DNA from the mapping populations. The same polymorphism was observed in both RAPD and SCAR analysis in two populations, Seafarer/UI-114 and G91201/Alpine. Nevertheless, SW13 amplification product differed from that of $OW13_{690}$ in the N84004/Michelite population. In this case, the *I* gene in three resistant plants was amplified by the SCAR marker but not by the RAPD marker (Table A1.2). These longer SCAR primers showed increased specificity and sensitivity when compared to the RAPD primers. Other reports also demonstrated

that longer primers were more specific and sensitive than RAPD, although in some cases they amplified an alternate allele from both parents and the original RAPD polymorphism was lost (Paran and Michelmore 1993), while in other reports RAPDs were converted into a codominant marker (Adam-Blondon et al. 1994, Timmerman et al. 1994, Horvath et al. 1995, Ohmori et al. 1996).

Table A1.2. Linkage analyses of common bean F_2 populations segregating for the molecular markers and the dominant *I* allele. Data for $OW13_{690}$ from Haley et al. 1994a.

Population	Expected ratio*	*I*/$OW13_{690}$		*I*/SW13	
		cM±SE	observed frequency	cM±SE	observed frequency
Seafarer/UI-114	9:3:3:1	1.3 ± 0.8	163:2:1:57	1.3 ± 0.8	154:2:1:55
N84004/Michelite	9:3:3:1	2.5 ± 1.1	140:5:0:51	1.0 ± 0.7	133:2:0:51
G91201/Alpine	3:6:3:1:2:1	5.0 ± 2.2	32:43:3:0:2:25	5.0 ± 2.2	31:43:3:0:2:25

*Expected ratios: 9:3:3:1, 9 *I*/- + marker: 3 *I*/- - marker: 3 *i*/*i* + marker: 1 *i*/*i* - marker; 3:6:3:1:2:1, 3 *I*/*I* + marker: 6 *I*/*i* + marker: 3 *i*/*i* + marker: 1 *I*/*I* - marker: 2 *I*/*i* - marker: 1 *i*/*i* - marker.

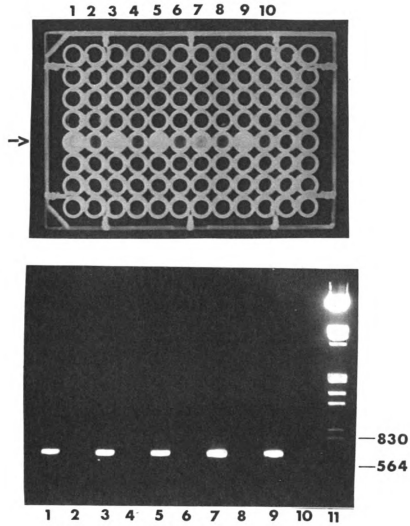

Figure A1.1. DNA amplification of ten bean cultivars using the SW13 SCAR marker. The PCR product were detected by direct ethidium bromide staining in microtiter plates (top) and by gel electrophoresis (bottom). (1) N84004 (*I/I*), (2) Michelite (*i/i*), (3) G91201 (*I/I*), (4) Alpine (*i/i*), (5) Seafarer (*I/I*), (6) UI-114 (*i/i*), (7) Chinook (*I/I*), (8) Sierra (*i/i*), (9) Montcalm (*I/I*), (10) negative control (no template DNA was added to the PCR reaction), (11) molecular weight marker (λ *Hin*d III/ *Eco*R I; size of bands indicated in base pairs).

Due to the dominant nature of the SW13 marker we were able to apply a quick minus/plus assay to detect the PCR product. As suggested by Gu et al. (1995), ethidium bromide was added to the amplification reaction. Strong fluorescence was observed in samples containing amplified DNA whereas weak fluorescence was observed in samples in which no fragment was expected. Results of all ethidium bromide assays were validated and confirmed by gel electrophoresis. Figure A1.1 shows an example of detection of SW13 product either in agarose gels or through directly staining in microtiter plates. Direct staining with ethidium bromide can be used in breeding programs when genotyping a large number of samples is required, to facilitate the development of more disease-resistant cultivars using marker-assisted breeding.

Use of different DNA polymerases in RAPD analysis has contributed to a lack of reproducibility across laboratories because each enzyme generates a specific banding pattern (Kelly 1995). Our results indicated that three different DNA polymerases can be used to demonstrate the expected polymorphism without any interference in the amplification of the *I* gene when SW13 primers were used (data not shown).

SW13 was tested in a collection of common bean genotypes representing both Andean and Middle American gene pools (Singh et al. 1991). In all cases, the presence or absence of the SCAR marker corresponded to the presence or absence of the *I* gene (Table A1.3), indicating broad utility of this marker across bean gene pools. In previous germplasm surveys of common bean, certain RAPD markers have been shown to be very useful as tools for indirect selection in populations derived from both gene pools (Haley et al. 1994b, Young and Kelly 1996), whereas the application of other markers was restricted to a specific gene pool (Miklas et al. 1993) or to a certain race within a gene

pool (Haley et al. 1993). In the present work, no gene pool or race specificity was observed. The SCAR marker SW13 is currently being used to pyramid the *I* gene with other epistatic recessive genes in the Michigan State University Bean Breeding Program for bean common mosaic virus resistance.

Table A1.3. Presence (+) or absence (-) of the *I* allele and the SW13 marker in bean genotypes from Andean and Middle American gene pools (Singh et al. 1991).

Gene Pools					
Middle American			Andean		
Genotype	*I* allele*	SW13	Genotype	*I* allele*	SW13
Alpine	-	-	A193	ND	-
Blackhawk	+	+	Chinook	+	+
Black Magic	+	+	Isabella	-	-
Bunsi	+	+	Kaboon	-	-
C-20	+	+	MDRK	-	-
Fiesta	-	-	Montcalm	+	+
Fleetwood	+	+	Perry Marrow	-	-
G91201	+	+	Ruddy	ND	+
Harofleet	+	+	Widusa	+	+
Harokent	+	+			
Mayflower	+	+			
Michelite	-	-			
N84004	+	+			
OAC Rico	+	+			
Raven	+	+			
Redkloud	+	+			
Seafarer	+	+			
Seaforth	+	+			
Sierra	-	-			
UI-114	-	-			

*Presence or absence of the *I* allele determined through inoculation with NL 3 strain of BCMV; ND=not determined.

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APPENDIX A2

GENETIC CHARACTERIZATION OF THE *Co-7* GENE PRESENT IN THE BEAN CULTIVAR G 2333

INTRODUCTION

The differential bean cultivar G 2333 possesses three independent dominant genes conditioning resistance to anthracnose (Chapter 2). Two of these genes were isolated in two different bean breeding lines derived from G 2333, SEL 1308 possesses the *Co-4*^{2} gene and SEL 1360 possesses the *Co-5* gene. The third resistance gene, *Co-7*, could not be isolated in a bean line because the *Co-4*^{2} gene is epistatic to the *Co-7* and no race of the pathogen that distinguishes these two genes has been identified. The objective of this study was to develop a bean line that carries only the *Co-7* gene in order to determine its usefulness in controlling anthracnose.

MATERIAL AND METHODS

The F_2 population SEL 1360 x G2333 was used to identify resistant genotypes carrying *Co-7* but not *Co-4²*. The segregating population was inoculated with race 521 to overcome the *Co-5* gene that is fixed in this population. In addition, the SAS13 marker (Young et al. 1998) was used to detect the *Co-4²* gene and select genotypes without the marker (Table A2.1).

Genotypes 1 through 8 are resistant and genotype 9 is susceptible when inoculated with race 521 (15R:1S segregation ratio). Genotypes 1 though 6 have the SAS13 marker linked to *Co-4²* (3:1 ratio for the presence and absence of the marker). We are interested on genotypes 7 and 8, where *Co-7* is present but *Co-4²* is absent and these genotypes were identified by selecting resistant plants lacking the SAS13 marker (Chapter 2). Those plants were selfed to distinguish between the homozygous and heterozygous for the *Co-7* gene.

Table A2.1 Nine genotypes segregating in the SEL 1360 x G 2333 F_2 population. Frequency, phenotype to race 521, and presence/absence of the SAS13 marker were determined.

Genotypes	Segregating Genes*	Frequency	Phenotype	SAS13 marker
1	*Co-4² Co-4² / Co-7 Co-7*	1	R	+
2	*Co-4² Co-4²/ Co-7 co-7*	2	R	+
3	*Co-4² Co-4² / co-7 co-7*	1	R	+
4	*Co-4² co-4² / Co-7 Co-7*	2	R	+
5	*Co-4² co-4² / Co-7 co-7*	4	R	+
6	*Co-4² co-4² / co-7 co-7*	2	R	+
7	*co-4² co-4² / Co-7 Co-7*	1	R	-
8	*co-4² co-4² / Co-7 co-7*	2	R	-
9	*co-4² co-4² / co-7 co-7*	1	S	-

*The *Co-5* gene is fixed in this population.

RESULTS

Using standard inoculation techniques and a linked molecular marker, a $F_{2:3}$ family homozygous for the *Co-7* (genotype 7) was identified and named SEL 111. Each F_3 seed was selfed and maintained separate. The $F_{3:4}$ families were inoculated with several anthracnose races (Table A2.2) and crossed to the susceptible cultivar Black Magic (BM). The derived F_2 population was screened with race 73 to confirm the presence of the two genes (*Co-5* and *Co-7*). The Black Magic/SEL 111 population unexpectedly segregated 3:1 (78R:29S) suggesting the presence of one gene. The data suggest that the *Co-7* gene must have been overcome by race 73 since *Co-5* is resistant to race 73. Support for this

hypothesis came indirectly from work conducted by Alzate-Marin et al. (1998) in Brazil. In an F_2 population derived from the cross Rudy (susceptible)/G 2333 inoculated with race 73, Alzate-Marin et al. (1998) observed a 15:1 ratio suggesting that G 2333 possessed only two independent dominant genes. The third gene in the population tested by Alzate-Marin et al. (1998), must have been defeated by race 73 and lends support to the observation herein reported.

Table A2.2 Disease evaluation on genotypes carrying anthracnose resistance genes derived from G 2333. Six plants of each genotype were inoculated.

	Race							
Genotype	7	73	449	521	1545	1929	2029	2047
G 2333	R*	R	R	R	R	R	R	R
SEL 1308	R	R	R	R	R	R	R	R
SEL 111	R	R	R	R	S	S	S	S
Black Magic	R	S	R	S	S	S	S	S

*R=resistant reaction, S= susceptible reaction.

DISCUSSION

Based on recent findings suggesting that more alleles exist at previously described locus, we did not discard the hypothesis that the *Co-7* gene (the third resistance gene of G 2333) could be an allele instead of a new locus. The two major resistance genes defeated by race 73 are the *Co-2* and *Co-3* genes. G 2333 does not possess the *Co-2* gene since markers linked to the *Co-2* are absent in G 2333 and the original F_2 population inoculated with race 521 at CIAT indicated the presence of genes other than *Co-2*, which is overcome by race 521. Since the third gene is resistant to race 521 and susceptible to race 73, the most likely candidate would be the *Co-3* gene or an allele at that loci. Current plans are underway to inoculate the same F_2 population with race 521 which overcome the *Co-5* and should permit the identification of the third gene, since neither are pathogenic on *Co-3*. In addition, to confirming the presence of two independent genes in SEL 111, the same population will be inoculated with a race which is ineffective against either the *Co-3* or *Co-5* genes.

In order to confirm the presence of the *Co-3* locus in SEL 111, the line was crossed to Mexico 222 (*Co-3*) and the F_2 population will be inoculated with a race that confers R/R reaction to both parents. Race 521 will be selected since it overcomes the *Co-5* gene, but not the putative *Co-3* gene in SEL 111. If SEL 111 and Mexico 222 prove to be allelic, then there should be no segregation regardless of whether the *Co-5* gene is present or absent. This would give us insights into the identity of the third gene in G 2333. Markers for the *Co-3* gene will need to be developed to confirm these results.

Markers for the *Co-6* gene need to be screened in G 2333 and SEL 111 to eliminate this resistance locus as a candidate for the third gene.

Preliminary results indicated that SEL 111 is susceptible to race 1929. If this is confirmed then the theory that the third gene in G 2333 is *Co-3* is unlikely unless SEL 111 carries a weaker allele at this locus. A weaker allele of the *Co-3* gene was reported (Fouilloux, 1979) in Mexico 227 (now extant). This allele can be discriminated from the original *Co-3* gene by race 31 (kappa). *Co-3* is resistant to race 31 whereas, the second allele is susceptible. Both alleles are susceptible to races 73 and 89, however, SEL 111 cannot be inoculated with race 31 to determine if it carries the *Co-3* resistant gene or the susceptible allele at the same locus, since SEL 111 also carries the *Co-5* gene which confers resistance to race 31.

In order to test this possibility, resistant F_3 progeny of the BM/SEL 111 population identified after inoculation with race 521 will be selected and screened against the marker linked to the *Co-5* gene to identify homozygous resistant individuals absent for the *Co-5* gene but carrying the third gene. Those individuals could be inoculated with race 31 and crossed with Mexico 222, known to carry the *Co-3* gene. Allelism tests will be conducted to confirm if the third gene in G 2333 is in fact the *Co-3* gene.

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APPENDIX A3

SCAR MARKER LINKED TO THE RUST DISEASE RESISTANCE GENE *UR-5* IN COMMON BEAN

INTRODUCTION

In common bean (*Phaseolus vulgaris* L.), resistance to several pathogens is controlled by major genes. Indirect selection for those genes would be greatly facilitated if tightly linked molecular markers were available. SCAR (sequence characterized amplified region) markers were chosen for selection purposes because they have proven to be reliable, reproducible, robust, and easily scored when a single polymorphic band is generated (Gu et al. 1995, Melotto et al. 1996). Here, the development of a SCAR markers linked to the major disease resistance gene *Ur-5* is reported. This gene conditions resistance to multiple races of bean rust caused by *Uromyces appendiculatus* (Pers.) Unger var. *appendiculatus* (Stavely 1984).

MATERIALS AND METHODS

The source for the gene *Ur-5* was the genotype B-190. Random amplified polymorphic DNA (RAPD) marker linked to this gene was previously identified $OI19_{460}$ (Haley et al. 1993). The amplification products were cloned and sequenced following procedures described by Melotto et al. (1996). SCAR primers were designed based on those sequences and specific PCR (polymerase chain reaction) files were created to amplify a single polymorphic band linked to the resistance gene of interest. Linkage between the SCAR marker and the resistance gene was calculated based on the recombination ratio observed in segregating populations.

RESULTS AND DISCUSSION

The SCAR marker developed, amplified a single polymorphic band of the same size as the correspondent RAPD marker. In Table A3.1, the molecular marker, the PCR file, and the linkage between the gene and the marker are specified. The SCAR primer sequences are shown in Table A3.2.

Table A3.1 Molecular marker, PCR file, and linkage between the *Ur-5* gene and the SI19 marker.

Gene	RAPD marker	SCAR marker	PCR file	Linkage
Ur-5	$OI19_{460}$	SI19	94°/10sec; 67°/40sec; 72°/2min. 34 cycles	0 cM*

*No recombinants were found.

Identification of linked SCAR markers is particularly useful for marker-assisted selection and can be readily used for locating resistance genes on linkage maps. The *Ur-5* gene has been mapped to linkage group B4 of integrated bean linkage map (Freyre et al. 1998). Resistance genes often present epistatic interactions and are difficult to combine with test crossing the progeny. The marker SI19 has been used to facilitate the pyramiding of *Ur-5* resistance gene into previously resistant bean germplasm (Stavely 1999) in order to achieve broad long-term resistance to the variable pathogens *U. appendiculatus*.

Table A3.2 SCAR primer sequences derived from the RAPD marker $OI19_{460}$ linked to the *Ur-5* gene in common bean.

Gene	SCAR marker	Primer sequence (5'-3')*	Primer length
Ur-5	SI19	AATGCGGGAGATATTAAAAGGAAAG	25-mer
		AATGCGGGAGTTCAATAGAAAAACC	25-mer

*Underlined sequence corresponds to the original 10-mer RAPD primer.

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