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INHERITANCE STUDIES AND DEVELOPMENT OF RAPD MARKERS FOR MAJOR ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

presented by

Roberto Antonio Young Bustillo

has been accepted towards fulfillment
of the requirements for

Doctoral degree in Crop & Soil Sciences - Plant Breeding & Genetics

James D. Kelly
Major professor

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INHERITANCE STUDIES AND DEVELOPMENT OF RAPD MARKERS FOR MAJOR ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

By

Roberto Antonio Young Bustillo

A DISSERTATION

Submitted to
Michigan State University
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Plant Breeding and Genetics Program
Department of Crop and Soil Sciences

1995

ABSTRACT

INHERITANCE STUDIES AND DEVELOPMENT OF RAPD MARKERS FOR MAJOR ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

By

Roberto Antonio Young Bustillo

The inheritance of the genetic resistance to bean anthracnose in genotypes, Catrachita and SEL 1360, derived from two differential cultivars, AB 136 and G 2333, is described. Segregation data indicated that a single dominant gene is responsible for the anthracnose resistance in Catrachita, and the gene was situated at a different locus from previously characterized resistance genes: *A*, *Are*, *Mexique 1*, *Mexique 2* and *Mexique 3*. It is proposed that the single dominant resistance gene present in Catrachita (AB 136) be assigned the gene symbol *Are 1024* corresponding to its ranking in the bean anthracnose differential cultivar series. Segregation data suggested that a single dominant gene was conditioning resistance to anthracnose in SEL 1360, and the gene is independent from the *A*, *Are*, *Mexique 1*, and *Mexique* 2 genes. However, the dominant gene in SEL 1360 did not segregate independently from the resistance gene *Mexique 3* in the differential cultivar TU, demonstrating that both dominant alleles are located at the same locus. Independent segregation of the two dominant genes was also observed between Catrachita and SEL 1360, indicating that these genes are independently inherited.

Six random amplified polymorphic DNA (RAPD) markers linked to the *Are*, *A*, *Are 1024* and *Mexique 3* loci were developed using near-isogenic lines, bulked segregant analysis, and heterogeneous inbred populations either singly or in combination. A RAPD marker designated $OQ4_{1440}$, was found tightly linked in coupling-phase with the *Are* gene. $OQ4_{1440}$, mapped at 2.0 ± 1.4 centimorgans (cM) from the *Are* allele in the Andean genetic background and at 5.5 ± 2.3 cM in the Middle American background. A second coupling-phase RAPD marker $B355_{1000}$, mapped at 5.4 ± 2.3 cM from the *Are* allele in the Andean genetic background and at 7.7 ± 2.7 cM in the Middle American background. Based on a recombination distance of 7.0 ± 1.9 cM between the two RAPD markers, $OQ4_{1440}$ and $B355_{1000}$ appear to flank the *Are* gene. The bracketing molecular markers allowed tagging of the *Are* allele with a selection fidelity of 99%. The $OF10_{530}$ RAPD was tightly linked in repulsion-phase to the *A* allele (1.9 ± 1.4 cM). A survey of bean genotypes for the presence or absence of the marker locus, revealed that $OF10_{530}$ RAPD would facilitate the introgression of the *A* gene across *Phaseolus* gene pools. A coupling-phase RAPD marker $OAH1_{780}$ and a repulsion-phase RAPD marker $OAK20_{890}$, cosegregated with alleles at the *Are 1024* locus in three different segregating populations. The $OAH1_{780}$ and $OAK20_{890}$ RAPD markers were found linked (12.3 ± 1.9 and 7.3 ± 1.5 cM, respectively) and flanked the *Are 1024* locus. Coupling and repulsion-phase RAPD markers when used as a codominant pair showed higher selection efficiency, for the identification of homozygous (*Are 1024 Are 1024*) F_2 individuals, as opposed to individual selection for either a coupling-phase or a repulsion-phase marker. The RAPD marker $OAB3_{450}$ cosegregated with the *Mexique 3* allele in two different populations. The $OAB3_{450}$ RAPD was found linked at 5.9 ± 1.7 in coupling with the *Mexique 3* allele.

Pyramiding four major anthracnose resistance genes using marker-assisted selection is now feasible in common bean, and the effective durability of the resistance to *C. lindemuthianum* could be increased if Andean and Middle American genes were combined.

DEDICATION

To my wife, Nane, with admiration and love.

To my children, Gabriel, Rebeca, Paulo Roberto and Luis Eduardo, I hope that this achievement will serve as an inspiration to give your best in life.

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I would like to express my sincere gratitude to all those individuals that in one way or another helped me to complete my doctoral program:

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To the Escuela Agrícola Panemericana, El Zamorano, for supporting my candidacy to come to MSU to pursue a Ph.D. degree.

Last but not least: To you Lord God, my strength and inspiration.

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GENERAL INTRODUCTION

Biotic stresses are among the main biological constraints affecting common bean (*Phaseolus vulgaris* L.), production worldwide. Bean anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lambs.-Scrib., is recognized as one of the most wide spread and economically important fungal diseases of common bean (Hubbeling, 1957; Goth and Zaumeyer, 1965; Zaumeyer and Meiners, 1975; Chaves, 1980; Pastor-Corrales and Tu, 1989). Anthracnose is a seed-transmitted disease and when conditions of high humidity (> 92%) and moderate temperatures (17-24 °C) are met, complete yield losses can be induced on susceptible genotypes (Zaumeyer and Thomas, 1957; Tu and Aylesworth, 1980; Pastor-Corrales and Tu, 1989).

The pathogen is capable of infecting different parts of the bean plant during all stages of growth. The initial symptom of anthracnose infection is a dark brown to black lesion with purplish red margins along the veins of the lower surface of leaves. Similar symptoms can be observed on leaf petioles and stems. On bean pods, symptoms appear as circular, reddish-brown lesions, which develop into sunken cankers where pink to flesh colored fruiting structures (acervuli) containing spores (conidia) of the fungus develop (Saettler and Hart, 1983). After invading the pod, mycelia and conidia of the fungus infect the seed coat and become established. Infected seeds are discolored and may develop brown to black cankers which can extend into the cotyledons, resulting in losses in yield,

and seed quality (Zaumeyer and Thomas, 1957; Saettler and Hart, 1983; Tu, 1992; Pastor-Corrales and Tu, 1989).

Pathogenic variation. The host range of *C. lindemuthianum* is largely restricted to bean (*P. vulgaris*), cowpea (*Vigna unguiculata*) and tepary bean (*P. acutifolius*) (Zaumeyer and Thomas, 1957). Within the *P. vulgaris* species, *C. lindemuthianum* has shown extensive physiological differentiation. The existence of pathogenic races was first observed by Barrus (1911), who distinguished two distinct isolates which differed in their ability to infect a group of bean genotypes. The two characterized biological strains were designated as alpha and beta (Barrus, 1918). Since then, the occurrence of other races has continuously been reported in the literature: race gamma (Burkholder, 1923), delta (Andrus and Wade, 1942), epsilon (Charrier and Bannerot, 1970), alpha-Brazil (Fouilloux, 1975), lambda (Hubbeling, 1976), kappa (Kruger et al., 1977), jota (Hubbeling, 1977). In general, much broader pathogenic variation of *C. lindemuthianum* has been observed in Mexico, Central America, to a lesser extent, the Andean region, than has been observed in Europe and North America (Beebe and Pastor-Corrales, 1991). For instance, in Mexico races are assigned to five distinct groups (Groups I, II, III, IV and alpha) based on their reaction on a set of genotypes. Using this differential set, Yerkes and Ortiz (1956), reported the existence of race alpha, beta and gamma and ten new races of the pathogen designated MA-1 through MA-10. Recently, Garrido-Ramírez and Romero-Cova (1989), reported the identification of eight new races, designated MA-23 to MA-30; adding to the collection of 22 previously described races of anthracnose in Mexico. In Central America, using the anthracnose differential cultivar set proposed by CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia) (CIAT, 1988; Pastor-Corrales, 1991), Rava et al.,

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(1993) reported the identification of seven different pathotypes, six of which were alpha-Brazil type while the remaining one belonged to the Mexican Group I. Pathogenic variation, in Brazil, was classified into groups of races: alpha, delta and Brazilian I and II; and individual races are identified as 'BA' followed by a number (Oliari et al., 1973; Pio-Ribeiro and Chaves, 1975). Seven physiologic races identified by Oliari et al., (1973) were classified as belonging to the Brazilian groups I and II, alpha group and Mexican group I. More recently, Menezes and Dianese (1988), identified races alpha, delta, epsilon, kappa and lambda and the new races zeta, eta, teta and mu, from a comprehensive collection of isolates from different parts of Brazil. Race characterization have also been conducted in Colombia where race beta, delta, kappa, alpha-Brazil, Mexico group II and new isolates not previously reported, were observed (Cobo-Soto, 1986; Pastor-Corrales and Tu, 1989). In Africa, Leakey and Simbwa-Bunnya (1972), reported five new races among 19 Uganda isolates. Races resembling alpha, beta, delta and gamma pathotypes were also observed in this country. Apart from beta and delta, gamma was the most abundant race observed in Malawi; where three new races of *C lindemuthianum* were also reported (Ayonoadu, 1974).

It is clear that worldwide *C. lindemuthianum* has demonstrated considerable variation in pathogenicity. However, comparisons of race characterization studies between regions becomes very difficult due to different sets of differential cultivars commonly used by researchers. Currently, bean researchers are using the established universal set of differential cultivars and the numerical system for classifying races (CIAT, 1988; Pastor-Corrales, 1991), to facilitate data exchange and breeding work. The proposed set of anthracnose differential cultivars is composed of 12 genotypes, each

differential cultivar has a fixed position in the general order of the set, and is identified with a numeric value. The genotypes and their binary values are: Michelite (1), Michigan Dark Red Kidney (2), Perry Marrow (4), Cornell 49242 (8), Widusa (16), Kaboon (32), Mexico 222 (64), PI 207262 (128), TO (256), TU (512), AB136 (1024), and G 2333 (2048). The numerical system (binary system) of nomenclature is used for classification of races, based upon the spectrum of pathogenicity exhibited by an isolate on the 12 differential genotypes. Values are assigned as the sum of the binary numbers of the differentials on which the isolate is pathogenic.

Host genetic resistance. Genetic resistance to *C. lindemuthianum* was first observed in the cultivar, Wells Red Kidney, which was later released for commercial production (Barrus, 1911, 1915). It was determined that the anthracnose resistance in Wells Red Kidney was governed by a single dominant gene which conferred resistance to races alpha and beta (Burkholder, 1918; McRostie, 1919). At present, five major anthracnose resistance genes have been genetically characterized. They include: *A* (McRostie, 1919), *Are* (Mastenbroek, 1960) and *Mexique 1, 2* and *3* (Fouilloux, 1979). However, more complex genetic systems responsible for the resistance to bean anthracnose have been observed in several studies. To explain the resistance to races beta and gamma, Andrus and Wade (1942) proposed a system of ten genes in three allelomorphic series, and gene interaction at three loci. Both duplicate and complementary genes and one dominant gene for susceptibility. Evidence suggesting duplicate factor loci and complementary factors with multiple allelism was observed to condition resistance to races alpha, beta and gamma (Cardenas et al., 1964). In addition, linkages between genes

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from distinct duplicate and complementary factor sets was also reported. Independent and complementary gene action at one or two different loci conferred resistance to races beta gamma and delta (Muhalet et al., 1981). An allelomorphic series of three alleles was also observed controlling resistance to race beta. It is noteworthy to indicate that studies on the genetics of the resistance may differ depending on the genotypes involved and/or the races of *C. lindemuthianum* used.

The single dominant *Are* gene which confers resistance to alpha, beta, gamma, delta, epsilon and lambda races (Pastor-Corrales and Tu, 1989) has been the source of resistance most widely utilized by plant breeders throughout the world (North America: Tu and Aylesworth, 1980; Muhalet et al., 1981; Tu, 1988, 1992. Europe: Mastenbroek, 1960; Goth and Zaumeyer, 1965; Fouilloux, 1979. Latin America: Menezes and Dianese, 1988. Africa: Leakey and Simbwa-Bunnya, 1972; Ayonoadu, 1974). Despite the broad resistance shown by the *Are* gene, numerous races have been observed to be pathogenic on *Are*, thus limiting its usefulness in Latin America (Menezes and Dianese, 1988; Pastor-Corrales and Tu 1989; Beebe and Pastor Corrales, 1991; Rava et al., 1993). Break down of *Are* resistance by the kappa race has also been reported in Europe (Krüger et al., 1977), and more recently races 73 and alpha-Brazil overcome the resistance in North America (Kelly et al., 1994a; Tu, 1994). In addition to race 73, Kelly et al. (1994a) also reported the first appearance of race 7 which was virulent on genotypes carrying the *A* resistance gene. Considering that most of the commercial cultivars in Michigan carry either *A* or *Are* genes, bean production in this state is under potential threat from these two new races. However, the combination of *A* and *Are* genes in a single genotype would afford

protection to all pathogenic variation of *C. lindemuthianum* currently observed in North America including races 7, 73 and alpha-Brazil.

Preventing an anthracnose epidemic in the case of a break down of *Are* resistance, bean breeders in Europe identified new sources of resistance to this disease. Three sources of resistance were found in a collection of Mexican bean germplasm, and the sources were characterized as monogenic, dominant genes independent from *Are*. These genes were described as *Mexique 1, 2* and *3* (Fouilloux, 1976; 1979). Among the European races of the pathogen, *Mexique 2* and *Mexique 3* genes conferred resistance to all known races, while the *Mexique 1* resistance was overcome by the alpha-Brazil race. In Latin America, Beebe and Pastor-Corrales (1991), reported that the *Mexique* genes were susceptible to fewer isolates of anthracnose than was the *Are* gene. These three major resistance genes have been included as members of CIAT's differential cultivars; Mexico 222 (*Mexique 1*), TO (*Mexique 2*) and TU (*Mexique 3*).

In recent years, a major effort to evaluate germplasm for anthracnose resistance has been undertaken by scientists at CIAT. Thousands of germplasm accessions from CIAT's gene bank were screened for new sources of anthracnose resistance using a diverse collection of isolates of the pathogen (Schwartz et al., 1982; Pastor-Corrales and Tu, 1989; Pastor-Corrales et al., 1995). Among the accessions that showed the broadest anthracnose resistance were AB 136 and G 2333 cultivars both members of CIAT's set of differential genotypes. The differential cultivar AB 136, is a small red seeded bean from Central America, where it has been used as a source of anthracnose resistance for cultivar development. Recently, a breeding line RAB 205 derived from AB 136, developed by CIAT, was released as the cultivar Catrachita in Honduras (Beebe and Pastor-Corrales,

1991)

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1991). Studies on the inheritance of the resistance of AB 136 were conducted by Gonçalves-Vidigal (1994). A series of crosses involving AB 136 and a group of differential cultivars (Michelite, Perry Marrow, MDRK, CN 49242 and PI 207262) were performed and segregating populations were evaluated using races alpha, delta and kappa in independent inoculations. A single factor was found responsible for the resistance to all three races. However, different genetic symbols (B, A', Q) were assigned to this factor to distinguished the resistance reaction to the different races used.

The final differential cultivar G 2333, originating as the Mexican cultivar Colorado de Teopisca, has shown the broadest race-specific resistance to *C. lindemuthianum*. Resistance has been confirmed in field tests in several countries and in greenhouse seedling tests in Colombia (Schwartz et al., 1982; Pastor-Corrales and Tu, 1989; Pastor-Corrales et al., 1994; 1995). Inheritance of the resistance in G 2333 was studied in a F_2 and backcross generations derived from a cross ICA Pijao/G 2333 (Pastor-Corrales et al., 1994). It was shown that duplicate and independent factors controlled resistance in G 2333 to race 521. Neither the study of Gonçalves-Vidigal (1994; AB 136) nor the study of Pastor-Corrales et al. (1994; G 2333), reported on the independency of the individual genes or the relationship of these dominant genes to other previously characterized resistance genes.

Disease control. Breeding for anthracnose resistance has played a major role in different parts of the world where the disease is a limiting production factor. However, genetic resistance should be incorporated as part of an integrated disease management

strategy, to increase the effectiveness of the control. For instance, anthracnose was once considered the most important disease in the bean-producing areas of the eastern U. S. (Zaumeyer and Thomas, 1957). However, the development of resistant varieties, improved seed production and certification programs significantly reduced the threat of this disease in this country (Saettler and Hart, 1983). An outbreak of anthracnose caused by the delta race in southern Ontario, demonstrated that sole dependence on genetic resistance to control this disease is not advisable (Tu and Aylesworth, 1980; Tu, 1981; 1994). As a result, control measures such as quarantine, pedigree seed inspection, seed treatment, cultural practices and breeding for disease resistance were initiated in Canada as part of a combined control strategy (Tu, 1992). It is noteworthy that in many parts of the world most of the disease management procedures already mentioned are difficult to implement. Breeding for resistance to anthracnose is the most practical and convenient strategy to pursue, assuming that resistance genes are available (Chaves, 1980; Silbernagel and Zaumeyer, 1973; Zaumeyer and Meiners, 1975; Schwartz et al., 1982).

Plant breeders recognized that breeding for vertical resistance against a highly variable pathogen such as *C. lindemuthianum*, is not the most desirable control strategy. This is because of the potential short term longevity of the reistance. Horizontal resistance, if available, seems to be more suitable for increasing the longevity of the resistance. However breeding for horizontal resistance as opposed to breeding for vertical resistance, is more complex since many genes might be involved and although selection for individuals with desirable agronomic and resistance traits might be possible, it would require a higher investment in time and resources. The use of multilines or cultivar mixtures to increase longevity will have limited acceptance in developed nations, since

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uniformity of the crop is usually required, by farmers, the processing industry and consumers. In some countries in Latin America and Africa, multilines or cultivar mixtures could have some usefulness, but if an organized seed industry is non-existent, multilines cannot be recommended. An easily adopted breeding strategy in most countries that might be suitable for developing cultivars with durable resistance against variable plant pathogens is gene pyramiding (Nelson, 1978). The concept of gene pyramiding is based upon the incorporation of two or more genes into a single cultivar. In common bean, combination of two or more vertical resistance genes has been previously suggested as a means to achieve stable resistance (Silbernagel and Zaumeyer, 1973). Currently, the development of DNA marker technology, has opened up the possibility of implementing gene pyramiding as a breeding strategy for the control of plant pathogens.

Molecular markers. The identification of individuals, which have accumulated a number of desirable agronomic/economic characteristics during the breeding process, constitutes the most important activity of a plant breeder. Several factors may interfere with the successful incorporation of an important trait(s): low heritability, genetic epistasis, undesirable linkages, pleiotropic effects, inadequate screening procedures. Molecular markers have gained the attention of breeders, since, they can overcome some of the limitations encountered by conventional selection procedures. Selection for a trait is performed indirectly based on the phenotype of a marker that cosegregates with the gene of interest. A useful marker(s) meets the following properties: easy recognition of all possible phenotypes, early expression in the development of the plant, neutral effect, free of epistatic effects (Stuber, 1991, Arús and Moreno-Gonzales, 1994). In addition to being

a useful breeding tool for indirect selection of a trait, molecular markers have been utilized in genetic fingerprinting to identify individuals, evaluate genetic relationships among individuals and study the genetic variation within and among populations (Caetano-Anollés et al., 1992; Smith and Chin, 1992; Thormann and Osborn, 1992; Nienhuis et al., 1994;)

Types of genetic markers. Markers can be classified into morphological, biochemical and DNA-based markers. Morphological trait markers have had limited application in plant breeding when used for indirect selection of a trait, because of undesirable phenotypic effects, unfavorable pleiotrophic interactions, dominance and late expression, rare polymorphisms and the potential effect on fitness or desirability of an individual (Tanksley 1983; Weeden, 1994a). The inherent properties that distinguish biochemical (isozymes, Tanksley and Orton, 1983) and/or DNA-based markers (RFLPs, Botstein et al., 1980; RAPDs, Welsh and McClelland, 1990; Williams et al., 1990) over morphological markers allows them greater utility in MAS breeding. Isozymes are based on genetic differences of specific enzymes coding for proteins that can be separated by electrophoresis. Isozymes, as opposed to DNA markers, are restricted by a limited number of markers available and by the number of biochemical assays needed to detect enzyme activity, which limits the number of genes able to be assessed (Stuber, 1991). The potential of isozymes as markers for indirect selection remains to be developed. Restriction fragment length polymorphisms (RFLPs) are based on genetic differences expressed as length polymorphisms in fragments resulting from the restriction of genomic DNA with endonucleases. Polymorphisms are detected by hybridizing labeled DNA clones to Southern blots containing DNAs digested with restriction endonucleases. RFLPs have

become widely used in the development of linkage maps of many crop species and for the mapping of genetic traits (Ganal et al., 1990; Tanksley et al., 1989). The RFLP assay, however, is expensive, laborious, requires the use of radioactivity, and thus is not readily adapted as a selection tool by most breeders working with large populations. Random amplified polymorphic DNA (RAPDs), a polymerase chain reaction (PCR)-based assay, detects nucleotide sequence polymorphisms within genomic DNA using a single short oligodeoxynucleotide of arbitrary sequence, resulting in the amplification of several discrete DNA products. The reaction products are separated on agarose gels and then visualized by ethidiun bromide staining. The RAPD protocol produces a dominant marker, because RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region. Polymorphisms are usually scored as the presence or absence of an amplification product from a single locus (Williams et al., 1990). The RAPD assay possess several advantages over RFLP technology for MAS application: no requirement of DNA sequence information, quick and simple to perform, uses fluorescence instead of radioactivity, nanograms quantities of DNA are required, and the technique is amenable to automation (Tingey et al., 1992).

RAPD markers for major disease resistance genes. The development of RAPD markers linked to major disease resistance genes has been successful in different crop species. For example RAPDs linked to major resistance genes have been identified in barley (*Hordeum vulgare* L., Barua et al., 1993; Borovkova et al., 1995), common bean (Adam-Blondon et al., 1994; Haley et al., 1993; 1994a; 1994b; 1994c; Johnson and Gepts, 1994; Johnson et al., 1994; 1995; Jung et al., 1994; Miklas et al., 1993; Young and Kelly,

1994), lettuce (*Lactuca sativa* L., Kesseli et al., 1992; Michelmore et al., 1991; Paran et al., 1991), oat (*Avena sativa* L., Penner et al., 1993), pea (Pisum sativum L., Weeden et al., 1994b), rice (*Oryza* sativa L., Mohan et al., 1994), tomato (*Lycopersicon esculentum* L., Klein-Lankhorts et al., 1991; Martin et al, 1991; Williamson et al., 1994), and wheat (*Triticum aestivum* L., Schachermayr et al., 1994).

Gene tagging strategies. The generation of near-isogenic lines (NILs) through backcrossing, offers the opportunity to find markers linked to the introgressed segment of the genome from the donor parent. In theory NILs are genetically identical except for a small region that contains the gene that differentiates the isogenic lines. Thus, polymorphisms observed between NILs represent putative linked markers to the gene of interest. Near-isogenic lines have been used in conjunction with RAPD markers to tag resistance genes in barley (Barua et al., 1993), common bean (Haley et al., 1994a; 1994b; 1994c) tomato (Klein-Lankhorts et al., 1991; Martin et al., 1991;), lettuce (Paran et al., 1991). The use of NILs, however, has the limitation of requiring a lengthy process to develop the contrasting isogenic lines. The generation of false polymorphisms, not associated with the gene of interest from the donor parent DNA segments even after several rounds of backcrossing to the recurrent parent is a problem (Young and Tanksley, 1989). Another strategy that overcomes some of the disadvantages of developing NILs was proposed by Michelmore et al. (1991). The method, called bulked segregant analysis (BSA), involves comparing two samples of DNA bulked from individuals in a segregating population which carry the same monogenic trait. All loci in the genome are regarded as being in linkage equilibrium except for the region of the genome linked to the selected locus. Polymorphic markers between the DNA bulks will be genetically linked to the loci

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determining the trait used to construct the bulks. Using BSA strategy, three RAPD markers were found linked to the *Dm/8* downy mildew resistance gene in lettuce (Michelmore et al., 1991). The *rpg4* gene in barley which confers resistance to pathotype QCC of *Puccinia graminis* f, sp. *tritici*, was tagged with three RAPD markers using BSA approach (Borovkova et al., 1995). In common bean, BSA has been used to develop markers linked to different rust (*Uromyces appendiculatus*) resistance genes (B-190, Haley et al., 1993; PI 181996, Johnson et al., 1995; Up_2, Miklas et al., 1993). The combination of NILs and BSA approaches have been used effectively for tagging disease resistance genes in barley (Barua et al., 1993), common bean (Haley et al., 1993; Johnson et al., 1995; Miklas et al., 1993), lettuce (Michelmore et al., 1991). A new method for targeting RAPD markers linked to disease resistance genes was proposed by Haley et al. (1994c). The method entails the development of a form of NIL through a process characteristic of line and cultivar development in many autogamous crop species. The use of such NILs served to identify RAPD markers linked to disease resistance genes in common bean (Haley et al., 1994a; 1994b; 1994c).

Application of RAPD markers. Marker-assisted selection (MAS) proposed by Melchinger (1990), could greatly facilitate the introgression of a gene(s) when direct selection for a phenotype is not practical or feasible. The application of MAS requires tight linkage (< 5 cM) between marker and gene of interest or the use of two loosely linked markers flanking the desirable locus (Tanksley, 1983). During the gene tagging process, coupling-phase as opposed to repulsion-phase markers are usually sought. However, Haley et al. (1994a) showed that selection against a repulsion-phase RAPD, as

opposed to selection for a coupling-phase RAPD, provided a greater proportion of homozygous resistance individuals, and a lower proportion of both segregating and homozygous susceptible genotypes. Further more, it was indicated that even a loosely linked (7.1 cM) repulsion-phase marker provided greater selection efficiency than a tightly linked (1.9 cM) coupling-phase marker. Selection efficiency was improved using a coupling and a repulsion-phase RAPD markers together as a codominant pair when compared to selection for either of the markers alone (Johnson et al., 1995). The identification of double recombinants (a homozygous individual with or without the marker locus) in a segregating population increased the selection efficiency of a codominant RAPD marker over a repulsion-phase RAPD marker. Johnson et al. (1995) indicated that the nature of the population with which the breeder is working, will determine the type of RAPD marker needed to implement efficient MAS. For instance, in BC_nF_2 and F_2 or later generation populations, selection against a RAPD marker linked in repulsion-phase to a dominant resistance allele, will discriminate between heterozygous and homozygous recessive genotypes from the homozygous dominant progeny. In contrast, in a traditional back cross breeding (BC_nF_1) population, coupling-phase RAPDs are necessary to obtain the heterozygous resistant individual using MAS. In a recombinant inbred line population, selection for a coupling-phase or against a repulsion-phase RAPD marker would have the same level of efficiency, since the level of heterozygosity would be minimal.

The large genetic variation observed in common bean is associated to two main centers of domestication, known as Andean and Middle American gene pools (Singh et al., 1991). Inter gene pool introgression of useful genes is commonly practiced among bean

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breeders. The usefulness of RAPD marker for MAS application in common bean, is best determined after selection for a marker linked to the gene of interest is practiced in different genetic backgrounds. Gene pool specific RAPD markers linked to rust resistance genes (PI 181996, Up_2) have been described (Johnson et al., 1995; Miklas et al., 1993). The most desirable marker is one which can be used for indirect selection of a resistance gene regardless of genetic background. Gene pool non-specific RAPD markers were found for the rust resistance B-190 gene (Haley et al., 1993) and the *I* gene for bean common mosaic virus (BCMV) resistance (Haley et al., 1994b). To overcome the limitation of gene pool specificity of a RAPD marker, Miklas et al. (1995), proposed the use of recombinant individuals as parents in MAS for disease resistance. For instance, a coupling-phase marker is selected against, when using a recombinant individual that is resistant and without the coupling RAPD, to introgress the gene into susceptible lines having the coupling marker. However, the implementation of this strategy can only be justified in those cases where an important major resistance gene needs to be introgressed into a particular genetic background which other wise would be impossible in its absence.

Some of the concerns about the applicability of RAPD technology in plant breeding are related to reliability or reproducibility of the assay in different laboratories. The reliability of RAPD markers was investigated by Weeden et al. (1992). Some aspects of the RAPD assay to consider were related to the purity and concentration of the DNA template, primer and Mg^{+2} concentration, and scoring errors due to contamination of template DNA and /or faintness of an amplified band. However, they concluded that the RAPD technique should be very useful for gene tagging. The problem of band reliability could be overcome with the use of sequence characterized amplified regions (SCARs)

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(Kesseli et al., 1992; Paran and Michelmore, 1993) or allele-specific associated primers (ASAPs) (Weeden et al., 1992). Both, SCAR and ASAP, are genomic DNA fragments at a single genetically defined locus that are identified by PCR amplification using a pair of single specific oligonucleotide primers (24-mers). These two molecular markers are advantageous over RAPD markers because they detect only a single locus and their amplification is less sensitive to reaction conditions. Since only one PCR-product is generated, post-amplification electrophoresis could be eliminated because the amplified DNA can be visualized by either staining with ethidium bromide (Gu et al., 1994), or by measuring DNA concentration in the reaction mixture using an ELISA reader or other rapid scanning devises capable of measuring absorbance at 260 nm (Weeden et al., 1992).

MAS in breeding for disease resistance. Durability of the genetic resistance in a released cultivar is a major concern of breeders working with variable plant pathogens. Nelson (1978), suggested pyramiding resistance genes as a strategy to provide durable resistance to plant pathogens. Gene pyramiding involves the accumulation in a single genotype of two or more resistance genes which are effective against the same strain of the pathogen. However, incorporating disease resistance genes using conventional breeding procedures could be laborious, time-consuming and expensive (Melchinger, 1990; Pedersen and Leath, 1988). One of the constraints of pyramiding genes is related to epistatic interactions between resistance genes. Since gene pyramiding requires that both epistatic and hypostatic genes be combined into a single genotype, breeders have no convenient way to select for the hypostatic gene without using multiple inoculations with different races or test crosses with a susceptible genotype (Kelly et al., 1993). In some

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instances, quarantine regulations may restrict the introduction of a strain(s) of the pathogen which is needed to select for an epistatic gene. Again, if a new strain(s) is introduced, it may required special manipulation and green house space (usually limited) to avoid escapes. The pyramiding of resistance genes can be greatly facilitated through MAS, providing markers tightly linked to the gene(s) of interest are available (Kelly, 1995; Melchinger, 1990; Stuber, 1991). In the absence of the pathogen, molecular markers allow the selection of a resistance gene based on the marker phenotype. Since RAPD markers are free of epistatic effects, the presence of a hypostatic gene can be identified indirectly by selecting directly for the linked marker. Pyramiding major resistance genes using MAS to increase the longevity of the resistance against variable plant pathogens has been suggested in barley (Barua et al., 1993; Borovkova et al., 1995), common bean (Kelly, 1993; 1995; Stavely et al., 1989), and wheat (Autrique et al., 1995). Currently, up to four major rust resistance genes (*Up_2*, *Ur-3*, B-190 and PI 181996) could be incorporated into a single common bean cultivar via MAS. Noteworthy is that the combination of *Up_2*, *Ur-3* and B-190 genes affords resistance to 63 of the 65 bean rust races characterized in the U.S. Dept. of agriculture collection (Kelly et al, 1994b). Similarly, complete protection to all known strains of BCMV is realized by combining the epistatic *bc-3* resistance gene with the hypostatic *I* gene in common bean. This combination is possible using MAS, since RAPD markers linked to both resistance genes are available (Haley et al., 1994a; 1994b).

Gene pyramiding and durable resistance. Attaining disease resistance that is both uniform against all current and future races of a pathogen is the primary objective of

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efforts to pyramid host genes. Several researchers have observed effective and stable resistance to plant pathogens and have attributed this lasting control to the combination of major resistance genes present in contemporary cultivars. For example, resistance to stripe rust (*Puccinia striiformis* West. var. *striiformis*) observed in several winter wheat cultivars grown in Western Europe during more than 15 years, suggested that the cause of the lasting resistance was due to the presence of three fully effective race-specific genes pyramided into single cultivars (Parlevliet, 1993). Similarly, Schafer and Roelfs (1985), indicated that the increase in durability of resistance to wheat stem rust (*Puccinia graminis* Pers. f. sp. *tritici*) in the northern plains was associated with multiple race-specific genes (6) pyramided into a single genotype. The successful control of rust by gene pyramiding also maybe related to eradication of the barberry (*Berbres vulgaris* L.) (Roelfs, 1982). Durability of the resistance was related to the low probability of mutation to virulence occurring at various loci in the pathogen population. The major assumption is that the pest lacks sufficient genetic variation to overcome multiple resistance. However, the mutability of the required virulence genes is only one aspect of the adaptation process of the pathogen population to an increased resistance level. Parlevliet and Zadocks (1977), suggested that another aspect is the exploitation of the newly produced virulence genes, since such genes can only spread through the pathogen when they increase the fitness of the population. During this exploitation phase an increase in gene frequencies of virulence genes from very low to high would be observed. Differences in longevity of resistance can therefore be expected to result from the inability of the pathogen population to produce the required virulence gene combinations, or from the inability to exploit the

gene or genes that are present in low frequencies, due to a reduction in pathogen fitness (Green and Campbell, 1979; Parlevliet and Zadocks, 1977; Van Der Plank, 1975). According to Person et al. (1976), a population large enough to maintain single mutants would be capable of producing recombinants by sexual or parasexual processes. Recombination of the virulence genes with high fitness is an essential part of the pathogen population adaptation process. The higher the recombination frequency, the easier new virulence genes can be incorporated in gene combination. Therefore, stability of the resistance is assumed to be highest when many resistance genes and so pathogenicity genes are involved, and when recombination in the pathogen is strongly restricted (Parlevliet and Zadocks, 1977). Other potential mechanisms responsible for the durability of resistance gene pyramids is the associated chance discovery of single resistance genes or combinations of small numbers of resistance genes that are more durable than average (Mundt, 1990, 1991). It would appear that breeding for gene pyramids will result in an increase of durability of resistance, regardless of the mechanism involved. However, there is no clear correlation between the number of resistance genes and the durability of the resistance (Mundt, 1991) which suggests that some gene combinations might be more effective than others. Green and Campbell (1979), indicated that stable resistance to stem rust in spring wheat was due to both the larger number and certain combinations of resistance genes. Johnson (1981, 1984), suggested that a large number of resistance genes deployed singly in a cultivar or genetic factors other than race-specific genes, might be involved in increasing durable resistance.

General objectives. Pyramiding major resistance genes into a cultivar has been proposed as an effective control strategy against variable plant pathogens. The accumulation of major race-specific genes into a genotype may increase the longevity of the genetic resistance. Marker-assisted selection offers the opportunity of gene pyramiding, since molecular markers used as breeding tools are able to overcome limitations of conventional breeding procedures. *Colletotrichum lindemuthianum* is a highly variable pathogen, and major anthracnose resistance genes incorporated singly into bean cultivars have been defeated by virulent races in different parts of the world. Traditionally pyramiding of major anthracnose resistance genes was not feasible, since molecular markers linked to resistance genes were not available. The general objective of this doctoral dissertation was to identify RAPD markers linked to major anthracnose resistance genes, and to propose breeding strategies to increase the effectiveness of gene pyramiding seeking durable anthracnose resistance in common bean. In order to achieve these goals, the research project was divided in three major stages, each with a specific objective. In Chapter one, the inheritance of the resistance derived from two differential cultivars, AB 136 and G 2333 to different races of *C. lindemuthianum* was the main objective. The relationship of the resistance factors in both differential cultivars to other previously characterized resistance genes was analyzed. Strategies for developing long-term durability of genetic resistance were also discussed. RAPD markers linked to the *Are* gene were sought in Chapter 2. Comparison of recombination distance between RAPD marker(s) and the *Are* allele cosegregating in two segregating populations (Andean and Middle American) were analyzed. In Chapter 3, research to identify RAPD markers

linked to three major anthracnose resistance genes, *A*, *Are 1024* and *Mexique 3*, is described. The implementation of RAPD markers in MAS for gene pyramiding, seeking durable resistance in common bean is discussed.

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Chapter One

CHARACTERIZATION OF THE GENETIC RESISTANCE TO *COLLETOTRICHUM LINDEMUTHIANUM* IN COMMON BEAN DIFFERENTIAL CULTIVARS

ABSTRACT.

The inheritance of genetic resistance to bean anthracnose in genotypes, Catrachita and SEL 1360, derived from two anthracnose differential cultivars, AB 136 and G 2333, is described. Segregation data from three different F_2 populations and their respective $F_{2:3}$ families indicated that a single dominant gene is responsible for the anthracnose resistance in Catrachita. In the test for allelism, Chi-square analysis confirmed that the single dominant resistance gene in Catrachita was situated at a different locus from previously characterized resistance genes: *A*, *Are*, *Mexique 1*, *Mexique 2* and *Mexique 3*. It is proposed that the single dominant resistance gene present in Catrachita (AB 136) be assigned the gene symbol *Are 1024* corresponding to its ranking in the bean anthracnose differential cultivar series. Segregation in the three F_2 populations where SEL 1360 was used as the resistant parent, fit a 3:1 (R:S) ratio and a 1:2:1 ratio in the $F_{2:3}$ families. Segregation data suggested that a single dominant gene was conditioning resistance to anthracnose in SEL 1360. The test for allelism involving SEL 1360 indicated that the single dominant gene in SEL 1360 is independent from *A*, *Are*, *Mexique 1*, and *Mexique* 2 genes. However, the dominant gene in SEL 1360 did not segregate independently from the resistance gene *Mexique 3* in the differential cultivar TU, demonstrating that both

dominant alleles are located at the same locus. Independent segregation of the two dominant genes in Catrachita and SEL 1360, was also observed. Deployment of major Middle American genes, such as *Are 1024* and *Mexique 3*, in different combinations with other characterized genes of Andean origin is possible and should contribute to more durable anthracnose resistance in common bean.

INTRODUCTION

Anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lambs.-Scrib., is one of the most wide spread and economically important fungal diseases of common bean (*Phaseolus vulgaris* L.). Complete yield loss can occur with susceptible genotypes when conditions favorable for the pathogen are present during the growing season (Zaumeyer and Thomas, 1957; Pastor-Corrales and Tu, 1989). Since *C. lindemuthianum* is a seedborne pathogen which exhibits extensive physiological variability, the use of genetic resistance has been the most effective control strategy implemented in Europe and North America (Fouilloux, 1979; Tu, 1992; Kelly et al., 1994a). In the U.S, the control of bean anthracnose by means of genetic resistance has relied primarily on the deployment of two genes, *A* and *Are* (Kelly et al., 1994a). The single dominant *A* gene was the first genetically characterized gene reported to confer resistance to race alpha (Burkholder, 1918; McRostie, 1919). The *Are* gene, which conditions resistance to six distinct physiological races of *C. lindemuthianum* (Tu, 1992), was described as a monogenic dominant factor by Mastenbroek (1960). In Europe, Fouilloux (1979) identified three additional single dominant resistance genes in a collection of Mexican germplasm. Allelism analysis showed that these Middle American genes segregated independently from each other and from the *Are* gene; and were designated *Mexique 1, Mexique 2, and Mexique 3*. Inheritance studies using the most common races (alpha, beta, delta, and gamma) and to a lesser extent, epsilon and kappa races, have been conducted. In addition to independent single dominant genes conditioning resistance to alpha (McRostie, 1919; Burkholder, 1918; Mastenbroek, 1960; Cardenas et al., 1964; Fouilloux, 1979; Del-Peloso et al., 1989; Gonçalves-Vidigal, 1994),

two independent complementary dominant genes have also been reported to confer resistance to the alpha race (Del-Peloso et al., 1989; Gonçalves-Vidigal, 1994; Young and Kelly see appendix). Resistance to races beta and gamma appears to be governed by the same dominant genetic factors (Fouilloux, 1979; Mastenbroek, 1960) in addition to a system of multiple alleles at two loci (Andrus and Wade, 1942; Cardenas et al., 1964; Muhalet et al., 1981). Both, independent and complementary gene action condition resistance to race delta (McRostie, 1919; Burkholder, 1918; Andrus and Wade, 1942; Mastenbroek, 1960; Cardenas et al., 1964; Fouilloux, 1979; Gonçalves-Vidigal, 1994), while, independent dominant alleles conferred resistance to races epsilon (Fouilloux, 1979) and kappa (Gonçalves-Vidigal, 1994).

Breeding for anthracnose resistance in North America has relied on the introgression of two major resistance genes, *Are* and *A*. Resistance break down, leading in some cases to disease epidemics, caused by races of *C. lindemuthianum* virulent on *Are* or *A* resistance genes have been observed in different parts of the world, including North America (Krüger et al., 1977; Tu,1981; Menezes and Dianese, 1988; Tu, 1994; Kelly et al., 1994a). Due to the potential short-term protection confered by single genes, a need for diverse sources of genetic resistance has been widely recognized among bean breeders. Using a diverse collection of isolates of the pathogen, scientists at CIAT, Colombia, screened several thousand germplasm accessions and identified new sources of genetic resistance (Schwartz et al., 1982; Pastor-Corrales and Tu, 1989; Pastor-Corrales et al., 1995). Two members of the differential set of cultivars, AB 136 and G 2333, used to categorize isolates of *C. lindemuthianum*, were consistently among the germplasm

accessions that showed the broadest resistance (Pastor-Corrales, 1991). Independent studies on the genetic characterization of these two differential cultivars have been conducted. According to Gonçalves-Vidigal (1994), a monogenic dominant factor present in AB 136 was responsible for the resistance to races alpha, delta and kappa, while two independent dominant genes conditioned resistance to race 521 in G 2333 (Pastor-Corrales et al., 1994). Neither study reported on the independency of the individual genes nor the relationship of these dominant factors to other previously characterized resistance genes.

In the present study, the inheritance of the resistance to different races of bean anthracnose in genotypes derived from two differential cultivars, AB 136 and G 2333, is described. The relationship of the resistance factors in AB 136 and G 2333 with other previously characterized resistance genes is analyzed. Finally, strategies for developing long-term durability of genetic resistance against bean anthracnose are discussed.

MATERIALS AND METHODS

Plant material. For the genetic characterization of the resistance in AB 136 and G 2333, three breeding lines derived from crosses involving these two genotypes were used in this study. A CIAT breeding line RAB 205 (BAT 1225/AB 136) released in Honduras as the cultivar Catrachita, was the source of the resistance from AB 136 (Beebe and Pastor-Corrales, 1991). CIAT breeding lines SEL 1360 and SEL 1308 (Talamanca * 2/G 2333), were utilized as the source of the resistance genes from the differential cultivar G 2333 (O. Voysest, personal communication). The derived genetic materials were used

because of their adaptation to growing conditions present at East Lansing. Synchronization of flowering for crossing purposes was facilitated using Catrachita and the SEL lines instead of the original parental genotypes which are photoperiod sensitive. Catrachita, SEL 1360 and SEL 1308 each were crossed to three Michigan cultivars; Black Magic (Kelly et al., 1987), Raven (Kelly et al., 1994b) and Blackhawk (Ghaderi et al., 1990). Black Magic (NEP-2/BTS) is susceptible to race 64 (alpha) and race 73; Raven (N84004/B85009) carries the *A* gene for resistance to races 64 and 73 but is susceptible to race 7; and Blackhawk (Tuscola/CN49-242//Black Magic/3/Midnight) carries the *Are* gene for resistance to races 7, 23 (delta) and 64 but is susceptible to race 73. During the present study, it was observed that SEL 1308 unlike SEL 1360 displayed a different resistance reaction when challenged with a wide group of races of the pathogen, suggesting that SEL 1308 carried a resistance factor(s) different from SEL 1360. The development of segregating populations for the characterization of the resistance in SEL 1308 is still under way and those findings will be reported elsewhere. A total of 17 segregating populations were developed for the genetic characterization of the resistance in Catrachita and SEL 1360. Six F_2 populations, three involving Catrachita (AB 136 resistance) and three involving SEL 1360 (G 2333 resistance) and their respective $F_{2:3}$ families, were used to determine the inheritance of the genetic resistance in both derived genotypes, while, ten F_2 populations were developed for the allelism test, using Catrachita and SEL 1360 in testcrosses with a series of genotypes carrying independently characterized genes previously described in the literature (Raven, Blackhawk, Mex 222, TO, and TU; which carry *A*, *Are*, *Mexique 1*, *Mexique 2* and *Mexique 3*, respectively;

Kelly et al., 1994b; Ghaderi et al., 1990; Fouilloux, 1979). The genetic independence of the new resistance sources was also evaluated in the cross of Catrachita/SEL 1360.

Preparation of races of C. lindemuthianum. Inoculum of each race used in the present study were obtained from monosporic cultures which had been grown and maintained in stocks of fungus-colonized filter paper for long-term storage. The identity of each race was first confirmed by the phenotypic reaction displayed with the anthracnose differential set (Pastor-Corrales, 1991). The numerical system used to identify different races is based on the sum of the binary values assigned to each of the 12 differential cultivars on which the specific race is pathogenic (CIAT, 1988). Parental genotypes, original (AB 136, G 2333) and derived (Catrachita, SEL 1360, SEL 1308) sources of resistance where screened with a collection of races of the pathogen (Table 1.1). Based on the different phenotypic reactions observed on these genotypes, specific races of *C. lindemuthianum* were chosen for the study of the genetic characterization of the resistance and different races were used for the allelism test (Table 1.2). Race 64 (ATCC 18987), race 23 (ATCC 18989) and race 73 (ATCC 96512) were selected to determine the inheritance of the resistance in Catrachita and SEL 1360 when crossed to the cultivars Black Magic, Raven and Blackhawk, respectively. Race 64 was selected to test for allelism in all segregating populations, except in Mex 222/Catrachita and Mex 222/SEL 1360 populations where race 7 (ATCC 96390) was used. A second independent inoculation, for the allelism test, was performed on TU/SEL 1360 F_2 population using race 7. Inocula of all races, except race 73 which was grown in Mathur's agar (Mathur et al., 1950), were prepared by placing fungus-colonized filter paper into Petri dishes containing potato-dextrose-agar. All cultures were incubated for 10 days under complete darkness at

Table 1.1 Phenotypic characterization of parental, original (AB 136, G 2333) and derived (Catrachita, SEL 1360, SEL 1308) sources of resistance for their reaction to different races of *C. lindemuthianum*.

	Race phenotypic reaction[z]					
Genotype	**7**	**23**	**64**	**73**	**1545**	**2047**
Black Magic	S	R	S	S	S	S
Raven	S	S	R	R	R	S
Blackhawk	R	R	R	S	S	S
Mex 222	R	R	S	S	R	S
TO	R	R	R	R	R	S
TU	R	R	R	R	S	S
AB 136	R	R	R	R	S	S
Catrachita	R	R	R	R	S	S
G 2333	R	R	R	R	R	R
SEL 1308	R	R	R	R	R	R
SEL 1360	R	R	R	R	S	S

[z] R = resistant; S = susceptible

Table 1.2 Crosses, generation and race of *C. lindemuthianum* used for the genetic characterization of the resistance in Catrachita (AB 136) and SEL 1360 (G 2333)

Cross		Generation[z]	Race[y]
Black Magic/Catrachita	(S/R)[x]	P, F_1, F_2, $F_{2:3}$	64
Raven/Catrachita	(S/R), (R/R)	P, F_1, F_2, $F_{2:3}$	23, 64
Blackhawk/Catrachita	(S/R), (R/R)	P, F_1, F_2, $F_{2:3}$	73, 64
Mex 222/Catrachita	(R/R)	P, F_1, F_2	7
TO/Catrachita	(R/R)	P, F_1, F_2	64
TU/Catrachita	(R/R)	P, F_1, F_2	64
Black Magic/SEL 1360	(S/R)	P, F_1, F_2, $F_{2:3}$	64
Raven/SEL 1360	(S/R), (R/R)	P, F_1, F_2, $F_{2:3}$	23, 64
Blackhawk/SEL 1360	(S/R), (R/R)	P, F_1, F_2, $F_{2:3}$	73, 64
Mex 222/SEL 1360	(R/R)	P, F_1, F_2	7
TO/SEL 1360	(R/R)	P, F_1, F_2	64
TU/SEL 1360	(R/R), (R/R)	P, F_1, F_2	64, 7
Catrachita/SEL 1360	(R/R)	P, F_1, F_2	64

[z] Parental, F_1, F_2 and $F_{2:3}$ families respectively. [y] Race of *C. lindemuthianum* used for the genetic characterization and the allelism test respectively. [x] Parental resistant (R) and/or susceptible (S) reaction to the races shown in column four.

24°C. Spore suspensions were prepared by flooding the plates with 5 ml of 0.01% Tween 20 in distilled water and scraping the surface of the culture with a spatula.

Disease phenotypic characterization. Parental material, F_1, F_2 and $F_{2:3}$ families were sprayed inoculated with a spore suspension (1.2×10^6 spores ml^{-1}) of the pathogen. The race of *C. lindemuthianum* used to characterize the resistance factors segregating in each cross and the respective generations tested is shown in Table 1.2. The protocol for spore inoculation was as follows: the first incompletely expanded trifoliate leaf of 15 to 18 day-old parental F_1 and/or F_2 plants was inoculated on the lower and upper leaf surfaces. Spore suspension was applied with a camel-hair paint brush. Conversely F_2 populations for the allelism test and $F_{2:3}$ families were spray inoculated with the spore suspension on the abaxial and stem surface of unifoliate leaves (10 to 12 day-old seedlings). Different inoculation methods were used because F_2 individual plants (particularly susceptible individuals), unlike $F_{2:3}$ plants and F_2 populations used for the allelism test, were saved for F_3 seed production . Inoculated plants were placed in a mist chamber at 100% RH, 22-25 °C for 48 hrs, then transferred to greenhouse conditions. Seven days post-inoculation disease phenotypic characterization was carried out. Disease reaction was rated on a 1-9 scale in which 1 = no visible symptoms and 9 = severely diseased or dead (Van Schoonhoven and Pastor-Corrales, 1987). Resistant (R) phenotype was assigned to plants with scores 1-3. Individual plants with scores 4-9 were considered susceptible (S).

RESULTS

Original and derived sources of anthracnose resistance were evaluated for their phenotypic reaction to a collection of races of the pathogen (Table 1.1). Different races (7, 23, 64, 73, 1545 and 2047) representing a wide range of virulence, were used to confirm if Catrachita and SEL lines were comparable to the anthracnose resistance present in the AB 136 and G 2333 differential cultivars. The cultivars Catrachita and AB 136 exhibited similar phenotypic patterns, both showed resistance to all races except races 1545 and 2047. None of the races tested were pathogenic on G 2333 and its derived line SEL 1308. However, SEL 1360 exhibited a different phenotypic pattern to the G 2333 parent. Two races, 1545 and 2047, overcame the resistance factor in this derived line. Since SEL 1308 and SEL 1360 were derived from G 2333 through backcrossing to the susceptible variety Talamanca, it is assumed that the resistance factor(s) observed in both lines came from the G 2333 parent. Since G 2333 carries two dominant resistance genes (Pastor-Corrales et al., 1994), it is possible that only one of the major genes was inherited in SEL 1360, thus a different phenotypic pattern was observed. Although similar disease reactions were observed between SEL 1308 and G 2333, it is not certain, in the absence of testcrosses, if SEL 1308 inherited both genes responsible for anthracnose resistance of G 2333.

Six F_2 populations, three involving Catrachita and three involving SEL 1360, and their respective $F_{2:3}$ families were used to determine the inheritance of the anthracnose resistance in different genetic backgrounds (Table 1.3). Eleven F_2 populations were

Table 1.3. Phenotypic characterization and observed ratio of F_2 progeny and $F_{2:3}$ families for their reaction to *Colletotrichum lindemuthianum*.

	Anthracnose reaction						
	F_2[y]			$F_{2:3}$[x]			
	No plants			No plants			
Cross[z]	R-	rr	P	RR	Rr	rr	P
Black Magic/Catrachita[w]	74	32	0.26	23	50	32	0.41
Raven/Catrachita[v]	76	34	0.19	28	47	34	0.26
Blackhawk/Catrachita[u]	79	32	0.41	23	55	31	0.55
Black Magic/SEL 1360[w]	84	27	1.00	29	54	27	0.95
Raven/SEL 1360[v]	89	22	0.25	35	51	21	0.14
Blackhawk/SEL 1360[u]	84	27	1.00	33	49	27	0.41

[z] Susceptible/Resistant crosses in all cases. [y] 3:1 (resistant, R- : susceptible, rr) and [x] 1:2:1 (resistant, RR : resistant Rr : susceptible, rr) expected ratio respectively; [w,v,u] phenotypic reaction to races 64, 23 and 73 of *Colletotrichum lindemuthianum* respectively.

utilized to test for allelism between the derived sources of resistance with other characterized genes (Table 1.4). The nature of the resistance in Catrachita (AB 136) and SEL 1360 (G 2333) was determined after analyzing the observed segregating ratios obtained from the disease phenotypic characterization of the three different F_2 populations and their respective $F_{2:3}$ families (Table 1.3). In the three crosses involving Catrachita as a parent (crossed with Black Magic, Raven and Blackhawk), the Chi-square test performed on data collected from the F_2 populations supported a fit to the 3:1 expected ratio of resistant to susceptible plants (R:S), respectively (Table 1.3). The Chi-square analysis performed on the $F_{2:3}$ families confirmed a 1:2:1 ratio in each F_2 population indicating that a single dominant gene is responsible for the anthracnose resistance in Catrachita.

The allelism study performed to test the independence of the single dominant resistance gene in Catrachita with the other five reported genes (*A*, *Are*, *Mexique 1*, *Mexique 2* and *Mexique 3*) is shown in Table 1.4. In all five F_2 populations, the observed segregation fit a 15:1 ratio (R:S), indicating that the single dominant gene conditioning resistance to anthracnose in Catrachita segregated independently from the *A*, *Are*, *Mexique 1*, *Mexique 2* and *Mexique 3* dominant genes.

Segregation in the three F_2 populations where SEL 1360 was the resistant parent, fit a 3:1 ratio of R to S respectively. A 1:2:1 ratio was confirmed in $F_{2:3}$ families from each F_2 population evaluated, suggesting that a single dominant gene was conditioning resistance to anthracnose in SEL 1360 (Table 1.3). Within four F_2 populations, segregation fit a 15:1 (R:S) ratio for the crosses of SEL 1360 with Raven, Blackhawk, Mex 222, and TO. This allelism test indicated that the single dominant resistance gene in SEL 1360 is independent and located at a different locus when compared to *A*, *Are*,

Table 1.4. Allelism test for the genetic characterization of the resistance to *Colletotrichum lindemuthianum* in Catrachita (AB 136) and SEL 1360 (G 2333).

	Anthracnose reaction			
	No. F_2 plants			
Cross[z]	R-	rr	X^{2} [y]	P
Raven/Catrachita[x]	114	3	2.12	0.15
Blackhawk/Catrachita[x]	119	14	3.45	0.06
Mex 222/Catrachita[w]	104	5	0.27	0.60
TO/Catrachita[x]	93	9	0.76	0.38
TU/Catrachita[x]	96	6	0.00	1.00
Raven/SEL 1360[x]	95	9	0.66	0.42
Blackhawk/SEL 1360[x]	69	4	0.00	1.00
Mex 222/SEL 1360[w]	96	6	0.00	1.00
TO/SEL 1360[x]	80	4	0.11	0.74
TU/SEL 1360[v]	102	0	5.78	0.02
Catrachita/SEL 1360[x]	91	11	2.85	0.09

[z] Resistant/Resistant crosses in all cases. [y] 15:1 (resistant, R- : susceptible, rr) expected ratio for two independently assorting genes; [x,w] phenotypic reaction to races 64 and 7 of *Colletotrichum lindemuthianum* respectively; [v] phenotypic reaction to two independent inoculations with races 64 and 7 of *Colletotrichum lindemuthianum* respectively.

Mexique 1 and *Mexique 2* genes (Table 1.4). However, no segregation was observed in the F_2 population derived from the cross between TU/SEL 1360 after two independent inoculations with races 64 and 7. This result indicated that the dominant gene in SEL 1360 is located at the same locus as the *Mexique 3* gene in TU. In addition, similar phenotypic reactions were also observed in the differential cultivar TU and SEL 1360 when evaluated with a group of anthracnose races (Table 1.1), supporting the finding of the allelism test that SEL 1360 and TU both carry the *Mexique 3* dominant allele.

The test of allelism performed on the F_2 population of the cross Catrachita/SEL 1360 segregated in a ratio of 15:1 ($P = 0.09$), indicating that the dominant resistance gene in Catrachita is independent from the resistance gene in SEL 1360 (Table 1.4).

DISCUSSION

Stable resistance to plant pathogens, such as *C. lindemuthianum* with extensive physiological variability, requires continual evaluation of germplasm and eventual introgression of diverse genetic resistance into commercial cultivars. The appearance of new races of anthracnose in Michigan (race 7 and 73; Kelly et al., 1994a) and Canada (alpha Brazil; Tu, 1994), which overcome *Are* (race 73 and Alpha Brazil) and *A* (race 7) genes, has forced breeders to seek alternative sources of resistance. Studies on the inheritance of anthracnose resistance in genetic material (Catrachita and SEL 1360) derived from AB 136 and G 2333 were conducted, because the broad base resistance observed in these differential cultivars could be of value in breeding for long-term

anthracnose resistance in beans (Schwartz et al., 1982; Menezes and Dianese, 1988; Pastor-Corrales and Tu, 1989; Pastor-Corrales et al., 1995). A unique monogenic dominant gene segregated in three different F_2 populations derived from crosses between resistant (Catrachita) and susceptible (Black Magic, Raven and Blackhawk) parents. It was concluded that this single dominant gene in Catrachita was responsible for the resistance to races 23 (delta), 64 (alpha) and 73. A coupling-phase RAPD marker tightly linked to the resistant allele in Catrachita was also present in AB 136 and absent in all susceptible parents (Black Magic, Raven and Blackhawk) (see Chapter 3), confirming that the single dominant resistance gene in Catrachita is the same resistance gene present in AB 136. A monogenic factor controlling anthracnose resistance in AB 136 was also described by Gonçalves-Vidigal (1994). In that work, segregating populations derived from crosses involving AB 136 as the resistant parent, were challenged with three different races of the pathogen. The symbols *B*, *A'* and *Q* were assigned to described a dominant gene governing the resistance to alpha, delta and kappa respectively. It is assumed that *B*, *A'* and *Q* symbols refer the same gene, since no experimental evidence suggests the contrary. Duplicate independent and dominant genes were observed to segregate in all populations derived from crosses involving Catrachita and each of the genotypes (Raven, *A*; Blackhawk, *Are*; Mex 222, *Mexique 1*; TO, *Mexique 2* and TU, *Mexique 3*) which carry a previously characterized resistance gene. In all instances, Chi-square tests confirmed a 15:1 ratio, suggesting that the single dominant gene in Catrachita was situated at a different locus from *A, Are, Mexique 1, Mexique 2* and *Mexique 3* resistance genes. In addition, independent segregation of two dominant genes was also observed in the cross

Catrachita/SEL 1360, indicating that these genes are located at different loci. However, Gonçalves-Vidigal, (1994) showed no segregation for susceptibility in progeny (F_2) derived from a cross between the differential cultivars PI 207262 and AB 136 when inoculated with races alpha, delta or kappa. She concluded that both cultivars carry a similar gene(s) each conditioning resistance to a different anthracnose race. Support for different alleles is based on the number of races which are virulent on PI 207262 and fail to overcome AB 136 resistance (Pastor-Corrales et al., 1994), or conversely, race 1545 was pathogenic on AB 136 but avirulent on PI 207262 (Table 1.1). In addition a RAPD marker linked to the AB 136 resistance allele was absent in PI 207262 (Chapter 3). Based on this evidence, AB 136 and PI 207262 would carry different alleles at the same locus.

The characterization of the genetic resistance in Catrachita (AB 136) determined that one dominant gene conditions resistance to bean anthracnose in this cultivar. The major gene in AB 136 is independent from the characterized genes: *A, Are, Mexique 1, Mexique 2 and Mexique 3*. In recognition of the fact that the *Are* gene, since it discovery and publication by Mastenbroek (1960), has been widely utilized by breeders as a source of resistance and extensively reported in the literature (gene tagging: Adam-Blondon et al., 1994; Young and Kelly, 1994; genetics: Fouilloux, 1979; Muhalet et al., 1981; breeding: Bannerot, 1971; Menezes and Dianese, 1988; Tu, 1992); the gene symbol *Are* has become synonymous with anthracnose resistance and is a descriptive symbol of that function. Therefore, it is proposed that the single dominant resistance gene present in AB 136 be assigned the genetic symbol *Are 1024* corresponding to its ranking in the differential series.

The resistance factor present in SEL 1360 was shown to be a monogenic dominant gene based on segregation ratios in three different F_2 populations. Support for a difference in inheritance between parent (G 2333) and offspring (SEL 1360) was expected since G 2333 and SEL 1360 displayed different phenotypic patterns after inoculation with anthracnose races 1545 and 2047 (Table 1.1). Considering that SEL 1360 is a selection derived from two backcrosses with the anthracnose resistant parent G 2333, it can be assumed that only one of the two dominant genes was transmitted to SEL 1360 offspring. The test for allelism involving SEL 1360 demonstrated that the segregation ratio in four populations (Raven, Blackhawk, Mex 222 and TO) followed a 15:1 (R:S) ratio indicating that the single dominant gene in SEL 1360 is independent from *A*, *Are*, *Mexique 1*, and *Mexique* 2 genes. However, the dominant gene in SEL 1360 did not segregate independently from the resistance gene (*Mexique 3*) in the differential cultivar TU indicating that both dominant alleles are located at the same locus. Since none of the anthracnose races tested (Table 1.1) could effectively differentiate between the two alleles, the data suggest that the resistance gene in SEL 1360 is the same dominant *Mexique* 3 gene present in TU. Additional evidence supporting this hypothesis comes from a coupling phase RAPD marker linked to the SEL 1360 allele which was also present in TU and the G 2333 parent (see chapter 3). Since G 2333 and TU belong to the same *Phaseolus* gene pool (Middle American, Singh et al, 1991), it is possible that these cultivars share common resistance genes. Based on the work of Pastor-Corrales et al. (1994), race 521 discriminates between the resistance gene in TU and the two genes present in G 2333. Results of the present study when compared with the results obtained

by Pastor-Corrales et al. (1994) suggest two possible genetic models. In the first model the dominant gene in SEL 1360 is the *Mexique 3* allele and is a third independent allele present in G 2333. This third allele was not detected by Pastor-Corrales et al. (1994) since it did not condition resistance to race 521 used in that study. The second model suggests that the dominant gene present in SEL 1360 is allelic to the *Mexique 3* gene and is one of the two dominant genes in G 2333 which condition resistance to race 521. Support for this model is lacking based on the RAPD marker evidence and in the absence of testing with race 521.

Two hypothesis can be formulated to explain the broad base resistance observed in SEL 1308 (Table 1.1). First, SEL 1308 carries both resistance genes from G 2333; and second, SEL 1308 inherited one of the major genes from G 2333 but is different from the gene in SEL 1360. This second gene alone is able to confer resistance to races 1545 and 2047. Since SEL 1308 was derived from G 2333 (two backcrosses) it is more likely that only one resistance gene was introgressed into this line as was the case with SEL 1360. The absence of the RAPD marker, linked to the SEL 1360 allele (*Mexique 3*), in SEL 1308 indicates that the resistance to bean anthracnose in this line is conferred by a different factor independent of the *Mexique 3* locus. Therefore, the resistance factor(s) in SEL 1308 becomes of major importance to breeders since they afford protection to the highly virulent races of the pathogen. The final characterization of the genetic resistance in SEL 1308 would distinguish between the proposed hypothesis.

Gene pyramiding has been suggested as a potential strategy for maintaining long-term durable disease resistance (Nelson, 1978). The accumulation of major resistance

genes into a cultivar would delay the appearance of new races of the pathogen. The basis for the stabilization of the resistance resides in the reduction in fitness of a pathogen when the number of virulence genes necessary to overcome host resistance increases (Van der Plank, 1968). The differential cultivar G 2333 constitutes an example of the effectiveness of gene pyramiding in controlling bean anthracnose when more than one major resistance gene is present in the host. A collection of 380 isolates of *C. lindemuthianum* from different parts of the world were avirulent on this genotype (Pastor-Corrales et al., 1994). To date, there has not been any isolate reported with the ability to overcome G 2333 resistance. Diversity in the sources of resistance used would also contribute to the stability of anthracnose resistance. For example, Kelly et al. (1994a) suggested that pyramiding *A* and *Are* genes would afford protection against all known anthracnose races in the U.S. It is noteworthy that the *A* gene is of Andean origin, in contrast to the *Are* gene of Middle American origin. The introgression of both Andean and Middle American genes seems to be an effective genetic combination for broad base resistance. For instance, Pastor-Corrales et al. (1994), reported that the *A* gene present in the differential cultivar Michigan Dark Red Kidney conferred resistance to a group of highly virulent races (73, 129, 133, 136, 385, 448, 521, 901, 905, 1409, 1473) of the pathogen. These races and race 1545 (Table 1.1) are pathogenic on most of the resistant sources of Middle American origin. It has been observed that some of the differential cultivars of Andean origin showed susceptibility to several isolates from South America whereas resistance to Middle American isolates was observed. In contrast, Middle American differential cultivars were usually immune to isolates of Andean origin (Pastor-Corrales et al., 1995).

Therefore, deployment of major genes, such as *Are 1024* and *Mexique 3*, in different combinations with other characterized genes such as the *A* gene should contribute to more durable anthracnose resistance in common bean.

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Chapter Two

RAPD MARKERS FLANKING THE *ARE* ANTHRACNOSE RESISTANCE GENE IN COMMON BEAN

ABSTRACT.

Incorporation of the Middle American and dominant *Are* gene, for bean anthracnose resistance, into commercial cultivars, has been the main disease control strategy adopted by breeders to effectively limit the potential damage of the pathogen. A random amplified polymorphic DNA (RAPD) marker designated $OQ4_{1440}$, generated by a 5'-AGTGCGCTGA-3' decamer primer, was found to be tightly linked in coupling with the *Are* gene. $OQ4_{1440}$ mapped at 2.0 ± 1.4 centiMorgans (cM) from the *Are* allele in the Andean genetic background and at 5.5 ± 2.3 cM in the Middle American background. A second coupling phase RAPD marker $B355_{1000}$ generated by a 5'-GTATGGGGCT-3' primer mapped at 5.4 ± 2.3 cM from the *Are* allele in the Andean genetic background and at 7.7 ± 2.7 cM in the Middle American background. Based on recombination distance of 7.0 ± 1.9 cM between the two markers, $OQ4_{1440}$ and $B355_{1000}$ RAPDs appear to flank the *Are* gene. Bracketing molecular markers allowed tagging of the *Are* allele with a selection fidelity of 99%. Use of the $OQ4_{1440}$ and $B355_{1000}$ RAPD markers for marker-based selection will afford the opportunity to retain the *Are* anthracnose resistance gene in bean germplasm, as other epistatic resistance genes are characterized, and incorporated into contemporary bean cultivars.

INTRODUCTION

Anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus.) Lams.-Scrib., is a serious seedborne fungus capable of inducing complete yield loss in susceptible bean (*Phaseolus vulgaris* L.) genotypes. Surveys in a wide range of production areas have identified several distinct races, suggesting extensive pathogenic variation on all continents (Pastor-Corrales and Tu, 1989). In North America, reported races include alpha, alpha Brazil, beta, gamma, delta, epsilon, and lambda in Canada (Tu, 1992, 1994). Recently, Kelly et al. (1994) identified two new races of *C. lindemuthianum* in the U.S. which are pathogenic on resistance genes currently deployed. The dominant allele at the *Are* locus, which confers resistance to alpha, beta, gamma, delta, epsilon, and lambda races of the fungus, was first found in a black bean from Venezuela, Cornell 49-242 (Mastenbroek, 1960) and has been used widely in North America and Europe as the main source of resistance against anthracnose (Fouilloux, 1978; Tu, 1992).

Marker-assisted selection (MAS), using molecular markers such as random amplified polymorphic DNA (RAPDs) as the selectable marker, has the potential to be a useful breeding tool in common bean, where monogenic disease resistance genes have been tagged (Adam-Blondon et al., 1994; Haley et al., 1993; Haley et al., 1994a, b, c; Johnson and Gepts, 1994; Johnson et al., 1995; Jung et al., 1994; Miklas et al., 1993). Conventional procedures for gene pyramiding are not practical for plant breeders due to epistatic interactions between resistance genes. Because pyramiding requires that epistatic and hypostatic genes be combined into a single genotype, breeders have no convenient way to select genotypes with a hypostatic gene without inoculating with different isolates

of a pathogen or test crossing back to a susceptible genotype. However the identification of molecular markers tightly linked to resistance genes allows indirect selection because the expression of the marker is not masked by epistatic interactions. The identification of a number of RAPD markers in common bean now permits gene pyramiding for disease resistance For example, RAPD markers can be used to pyramid three rust (*Uromyces appendiculatus*) resistance alleles (Up_2, *Ur-3*, B-190; Kelly, 1995) with other epistatic resistance alleles from the plant introduction collection (Johnson et al., 1995). Similar pyramiding opportunities for MAS have been suggested in breeding for resistance to bean common mosaic virus (BCMV), since the *I, bc-3* gene combination affords resistance to all known strains of BCMV (Haley et al., 1994b). Efficiency in selection can be improved by using a second marker either linked in repulsion with the resistance gene (Haley et al., 1994a) or linked in coupling but flanking the allele of interest (Tanksley, 1983).

Two gene pools exist in *P. vulgaris*, the Middle American and the Andean (Gepts and Bliss, 1985; Gepts et al., 1986; Singh et al., 1991) and crosses between these have been utilized to exploit breeding for anthracnose resistance in different bean seed and pod types. RAPD markers linked to genes introgressed from germplasm derived from a different gene pool appear to have lower recombinational frequencies and apparent tighter linkages than recombinational distances measured between the same RAPD linked to the resistance gene in the same gene pool (Haley et al., 1994b). Brown et al., (1989) demonstrated that linkage between traits can vary depending on the populations studied and the genetic similarity between parents. Because recombinational frequencies may be underestimated in wide crosses (Paterson et al., 1990), map distances based on figures

from such crosses may not represent the actual distance between markers and genes in more related genetic backgrounds with which breeders most frequently work.

The objectives of the present research were to find a RAPD marker linked to the Middle American *Are* allele, and compare its utility with another previously characterized marker ($B355_{1000}$) in Andean and Middle American bean populations. Comparison of recombinational distance between RAPD marker(s) and the *Are* allele in common bean gene pools were analyzed.

MATERIALS AND METHODS

Genetic material. Two groups of nearly-isogenic lines (NILs) were used to screen for RAPD markers linked to the Middle American *Are* gene in different genetic backgrounds. A pedigree generated (F_{11}) inbred line (designated K86002) heterogeneous for the *Are* locus was the source of NILs in the Andean genetic background. The kidney bean line K86002 was originally derived from introgression of the *Are* gene through backcrossing (CN49242/4*Montcalm//Charlevoix). The development of NILs from advanced heterogeneous lines has been described previously (Haley et al., 1994c). The DNA from K86002 lines, homozygous dominant and homozygous recessive for the *Are* locus respectively, constituted the contrasting NIL pair in the Andean genetic background. The other NIL pair was obtained from a F_2 population (A4512) derived by crossing two nearly-isogenic Middle American cultivars (Ex Rico 23 / OAC Rico; Beversdorf, 1983), which differed for the presence (OAC Rico) and absence (Ex Rico 23) of the *Are* gene. Bulked segregant analysis (Michelmore et al., 1991) was used to prepare the DNA bulk-NILs from the A4512 population in the Middle American genetic background. The two

contrasting DNA pairs (one pair Andean and one pair of Middle American origin) were screened with random decamer primers to identify RAPD markers linked to the *Are* allele. Progeny of 108 F_2 (103 $F_{2:3}$) individuals from the Andean (K86002) and 109 F_2 (104 $F_{2:3}$) individuals from the Middle American (A4512) crosses were used as mapping populations.

Disease evaluations. Parental material used as sources of DNA for primer screening were first genotyped for the *Are* locus by inoculating with the appropriate race of *C. lindemuthianum.* The choice of different races of *C. lindemuthianum* to screen for the *Are* resistance gene was based on differential disease reaction of germplasm from each gene pool. To discriminate effectively between resistant and susceptible genotypes in the segregating F_2 and $F_{2:3}$ populations, the beta race was used to screen the K86002 population and delta race was used to screen the A4512 population. Progeny tests of at least 11 to a maximum of 18 plants from each $F_{2:3}$ family from both mapping populations were carried out to confirm the disease expression of F_2 individuals and separate homozygous *AreAre* from heterozygous *Areare* genotypes. All pathogen inoculations were conducted using a suspension of 1.2×10^{-6} spores ml^{-1}. The first incompletely expanded trifoliate leaf of 15 to 18 day old F_2 plants was inoculated on the lower and upper leaf surfaces. Spore suspension was applied with an artist's camel-hair paint brush. Conversely, $F_{2:3}$ families were spray inoculated with the spore suspension on the abaxial and stem surface of unifoliate leaves (10 to 12 day old seedlings). Different inoculation methods were used because F_2 (particularly susceptible individuals), unlike $F_{2:3}$ plants, were saved for F_3 seed production and leaf tissue sampling for DNA extraction. Inoculated plants were placed in a mist chamber (100% RH) at 22-25 °C for 48 hr, then transferred to the greenhouse. Disease ratings were made 7 days post-inoculation.

Disease reactions were recorded as resistant (R) for those plants with no visible disease symptoms of anthracnose, and susceptible (S) for those plants with presence of enlarged lesions or sunken cankers on the lower side of the leaf or hypocotyl.

RAPD analysis. Prior to inoculation with *C. lindemuthianum*, tissue was collected for DNA extraction from young primary leaves (4 to 7 days post-emergence) from greenhouse grown plants. Two discs of leaf tissue were cut by clipping the leaf with a 1.5 ml microcentrifuge tube. A simple and quick extraction method developed by Afanador et al., (1993) was used for DNA extraction. The extracted DNA was standarized to a uniform concentration (10ng ul^{-1}) using DNA fluorometry (Hoefer TKO 100, Hoefer Scientific, San Francisco, Calif.).

The Polymerase Chain Reaction (PCR) cycling profile consisted of 3 cycles of 1 min/94°C, 1 min/35°C, 2 min/72°C; 34 cycles of 10 s/94°C, 20 s/40°C, 2 min/72°C; 1 cycle of 5 min/72°C; 1 s "Auto-segment Extension" (for extension phase of 34-cycle portion of PCR). Amplification was carried out in a Perkin Elmer Cetus DNA Thermal Cycler 480. Description of the PCR reactions have been reported previously (Haley et al., 1993; Miklas et al., 1993). Approximately 10 ng of genomic DNA template and 10 ng of a decamer primer (Operon Technologies, Alameda, Calif.) were combined in an 18.8 ul reaction, containing 2 units of Stoffel Fragment Polymerase (Perkin Elmer CETUS, Norwalk, Conn.). Amplified DNA fragments were resolved by electrophoresis in 1.4% agarose gel containing 0.5 ug ml^{-1} ethidium bromide, 40mM Tris-acetate, and 1 mM EDTA. Polymorphisms were scored as either presence or absence of a band (visualized using UV light) between bulked DNA pairs with or without the *Are* allele, respectively.

Prior to screening the mapping populations, DNA from other known resistant and susceptible genotypes were used to confirm potential RAPD-*Are* locus linkages.

Linkage with a second RAPD marker, identified by Weeden (personal communication) in recombinant inbred populations of Andean snap bean germplasm segregrating for the *Are* gene, was studied in the same two F_2 populations K86002 and A4512. The second RAPD marker was obtained using the B355 primer 5'-GTATGGGGCT-3' which amplified a 1000 bp fragment linked in coupling with the *Are* allele. The marker symbol B355 is the identification used by the University of British Columbia to describe the 10-mer primer. The RAPD marker $B355_{1000}$ was identified using the PCR protocol for DNA amplification recommended by GibcoBRL. The cycling profile consisted of 35 cycles of 1 minute each / at 92°, 35°, and 72°C using 1.0 unit of Taq DNA polymerase (GibcoBRL) as the thermal stable enzyme.

A simple dominant transmission of the *Are* gene and the putative linked RAPD markers were analyzed by Chi-square. The recombination frequency between a RAPD marker and the *Are* allele and between $OQ4_{1440}$ and $B355_{1000}$ RAPDs was calculated using Linkage-1 (Suiter et al., 1983) and Mapmaker (Lander et al., 1987).

RESULTS

A total of 346 random decamer primers were screened against the contrasting DNA combinations of resistant and susceptible genotypes from Middle American and Andean origin. Polymorphisms within at least one of the DNA pairs, were generated by approximately 32% of the primers, while only 8% of the primers tested produced polymorphic loci in both DNA pairs. The number of false positives generated between DNA pairs alone was relatively high, but once they were compared to DNA controls, the majority of them were eliminated. RAPD variability generated within the Middle American (A4512) bulk-NILs was less frequent (13%) than that found within the Andean (K86002) NILs (19%). Different levels of polymorphisms within Middle American versus within Andean gene pools have been reported by Haley et al., (1994d).

Identification of RAPD markers. Five putatively linked RAPD markers, two apparently associated in coupling and three in repulsion phase with the dominant *Are* allele, were identified across both populations. These RAPDs were assayed for co-segregation with F_2 genotypes characterized as resistant and susceptible in the two segregating populations Only one RAPD marker, generated by a 5'-AGTGCGCTGA-3' decamer primer and designated $OQ4_{1440}$,- 'O' for the source of the primer (Operon Technologies), Q4 for the specific kit (Q) and primer number (4), and $_{1440}$ for the size (bp) of the polymorphic fragment; was found to be tightly linked with the *Are* allele. The $OQ4_{1440}$ mapped at 2.0 ± 1.4 centiMorgans (cM) in the Andean genetic background and at 5.5 ± 2.3 cM in Middle American background from the *Are* allele (Table 2.1). A second coupling phase RAPD marker $B355_{1000}$ mapped at 5.4 ± 2.3 cM in the Andean genetic background and at 7.7 ± 2.7 cM in the Middle American background from the *Are* allele.

Table 2.1. Two-point chi-square (X^2) analyses and linkage estimates for marker loci ($OQ4_{1440}$ and $B355_{1000}$) and the anthracnose resistant *Are* allele.

Population[y]	Loci tested	Expected ratio[z]	Observed frequency	X^2	Probability	cM (r ± SE)
Middle American	*Are*/ $OQ4_{1440}$	3:6:3:1:2:1	29:39:0:1:4:31	83.7	0.00	5.5 ± 2.3
Andean	*Are*/ $OQ4_{1440}$	3:6:3:1:2:1	26:54:1:0:1:21	91.5	0.00	2.0 ± 1.4
Middle American + Andean	*Are*/ $OQ4_{1440}$	3:6:3:1:2:1	55:93:1:1:5:52	173.7	0.00	3.9 ± 1.4
Middle American	*Are*/ $B355_{1000}$	3:6:3:1:2:1	29:41:4:1:2:27	73.0	0.00	7.7 ± 2.7
Andean	*Are*/ $B355_{1000}$	3:6:3:1:2:1	27:53:4:0:1:18	74.7	0.00	5.4 ± 2.3
Middle American + Andean	*Are*/ $B355_{1000}$	3:6:3:1:2:1	56:94:8:1:3:45	147.9	0.00	6.6 ± 1.8
Middle American	$OQ4_{1440}$/ $B355_{1000}$	9:3:3:1	67:2:7:28	67.25	0.00	8.3 ± 2.9
Andean	$OQ4_{1440}$/ $B355_{1000}$	9:3:3:1	80:1:4:18	74.68	0.00	5.4 ± 2.3
Middle American + Andean	$OQ4_{1440}$/ $B355_{1000}$	9:3:3:1	147:3:11:46	141.60	0.00	7.0 ± 1.9

[y]Anthracnose evaluation of Middle American (Ex Rico 23/OAC Rico, A4512), and Andean (K86002 S/ K86002 R) populations conducted on $F_{2:3}$ progenies. [z]based on 1:2:1 genotypic segregation ratio for the *Are* locus and 3:1 ratio for the $OQ4_{1440}$ and $B355_{1000}$ RAPD markers.

Based on recombination distances of 7.0 ± 1.9 cM between the two markers across both gene pools (5.4 ± 2.3 cM in the Andean population, and 8.3 ± 2.9 cM in the Middle American population), $OQ4_{1440}$ and $B355_{1000}$ RAPDs appear to flank the *Are* allele (Table 2.1). Differences in recombination values were observed between populations, but these were not statistically significant ($P \leq 0.05$) for either marker. In each population, segregation ratios consistent with dominant monogenic inheritance were observed for the $OQ4_{1440}$ and $B355_{1000}$ RAPD markers and the dominant *Are* allele (Table 2.2). The amplification of the 1440 bp DNA fragment by the $OQ4_{1440}$ RAPD marker, was scored across a number of individuals with known genotype at the *Are* locus (Fig. 2.1). The presence and absence of the RAPD marker was associated with resistant and susceptible phenotypes of F_2 individuals and commercial cultivars possessing or lacking the *Are* gene respectively.

Selection efficiency. Based on actual observations, the efficiency of indirect selection for the *Are* locus using $OQ4_{1440}$ and $B355_{1000}$ RAPD markers was analyzed within and across gene pools (Table 2.3). Selection efficiency is defined here as the ability of a RAPD marker, used alone or a combination of both markers, to effectively distinguish between resistant (*AreAre* and *Areare*) and susceptible (*areare*) F_2 individuals based on the marker phenotype as selection criteria. Since $OQ4_{1440}$ RAPD mapped tighter to the *Are* allele than the $B355_{1000}$ RAPD, efficiency of indirect selection for resistant and susceptible F_2 individuals was higher when the $OQ4_{1440}$ marker was used alone regardless of genetic background (95.2 vs. 93.3% in Middle American and 98.1 vs. 95.1% in Andean genetic background). However when both RAPD markers were utilized together, selection efficiency within and across gene pools increased to 98.6% in the combined

Middle American and Andean populations. The largest improvement in selection efficiency was observed using $B355_{1000}$ alone (93.3%) when compared to the combination of $OQ4_{1440}$ + $B355_{1000}$ RAPDs (98.1%) in the Middle American genetic background.

Table 2.2 Chi-square (X^2) analysis for $OQ4_{1440}$ and $B355_{1000}$ RAPD markers and the *Are* allele segregating in Middle American and Andean F_2 and $F_{2:3}$ populations.

Population†	Locus tested	Generation	Expected Ratio	Observed Frequency	X^2	Probability
Middle American	*Are*	F_2	3:1	78:31	0.52	0.47
Middle American	*Are*	$F_{2:3}$	1:2:1	30:43:31	3.14	0.21
Andean	*Are*	F_2	3:1	86:22	1.00	0.32
Andean	*Are*	$F_{2:3}$	1:2:1	26:55:22	0.79	0.67
Middle American	$OQ4_{1440}$	F_2	3:1	73:36	3.33	0.07
Middle American	$B355_{1000}$	F_2	3:1	79:30	0.25	0.62
Andean	$OQ4_{1440}$	F_2	3:1	86:22	1.00	0.32
Andean	$B355_{1000}$	F_2	3:1	89:19	2.78	0.10

† Middle American = Ex Rico 23/OAC Rico (A4512); Andean = K86002 S/K86002 R

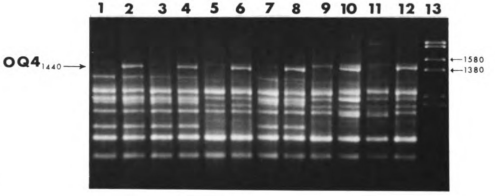

Figure 2.1 Amplification of genomic DNA using $OQ4_{1440}$ RAPD marker. Key to individuals: (1) anthracnose-susceptible Andean parent, (2) anthracnose-resistant Andean parent, (3) susceptible Andean F_2 individual, (4) resistant Andean F_2 individual, (5) anthracnose-susceptible Middle American DNA bulk, (6) anthracnose-resistant Middle American DNA bulk, (7) susceptible Andean cultivar (Montcalm), (8) resistant Andean cultivar (Isles), (9) anthracnose-susceptible Middle American parent (Ex Rico 23), (10) anthracnose-resistant Middle-American parent (OAC Rico), (11) anthracnose-susceptible Middle American cultivar (Mayflower), (12) anthracnose-resistant Middle American cultivar (Harokent), (13) molecular weight marker (l Hind III / EcoR1; size of bands indicated in bp).

Table 2.3 Efficiency of RAPD marker(s) linked to the *Are* allele for marker-based selection within and across common bean gene pools.

RAPD Marker(s)	Gene Pools[z]					
	MA		A		MA + A	
	No. of recomb.	Selection efficiency (%)	No. of recomb.	Selection efficiency (%)	No. of recomb.	Selection efficiency (%)
$OQ4_{1440}$	5	95.2	2	98.1	7	96.6
$B355_{1000}$	7	93.3	5	95.1	12	94.2
$OQ4_{1440} + B355_{1000}$	2	98.1	1	99.0	3	98.6

[z] MA= Middle American (Ex Rico 23/OAC Rico, A4512); A= Andean (K86002 S/K86002 R).

DISCUSSION

Identification and usefulness of RAPD markers. In plant breeding, marker loci tightly linked to genes of interest are needed for implementation of MAS strategies for gene introgression. The most desirable markers are those that exhibit tight linkage (≤ 5 cM; Tanksley, 1983) with the resistance genes. However, many studies restrict the application of markers to specific genetic population, so the usefulness of a linked marker is not always demonstrated across a range of genetic backgrounds. In common bean, RAPD markers may be gene pool specific and of limited value in selecting for resistance genes in the same genetic background from which the genes are indigenous. This limitation was demonstrated with the RAPD marker linked to the Up_2 gene which could not be used for selection for rust resistance in the Andean gene pool from which the Up_2 gene originated (Miklas et al., 1993). Therefore the value of that marker was restricted to the Middle American gene pool. This result is in contrast to that for the RAPD marker $OI19_{460}$ linked to the Middle American B-190 gene which can be used in all gene pools regardless of origin of the resistance gene (Haley et al., 1993). Similarly RAPD markers associated with the rust resistance gene *Ur-3* (Haley et al., 1994c) and the *I* gene for BCMV resistance (Haley et al., 1994b) have broad application across gene pools and genotypes. A RAPD marker found linked to the *Are* allele in an Andean snap bean population was reported by Adam-Blondon et al. (1994). Since this molecular marker was not tested across different genetic backgrounds its usefulness for introgressing the *Are* gene into Middle American germplasm using MAS remains to be confirmed.

The use of DNA from the two distinct genetic populations (Andean and Middle American) in the initial screening of primers, enabled us to identify a gene pool non-specific RAPD marker ($OQ4_{1440}$) for the *Are* gene. The second RAPD marker ($B355_{1000}$) linked in coupling with *Are* allele, exhibited similar differential recombination between the two populations. The linkage distances were greater than those found for the $OQ4_{1440}$ marker but since the differences were non significant, the $B355_{1000}$ marker is equally robust as a tool for indirect selection in populations of bean derived from the two gene pools. The linkage distance between the two markers is greater than the distance of either individual marker to the *Are* gene in both populations. Although the actual linkage distance is less than the sum of the individual distances between individual markers and the gene, the data clearly suggest that the markers flank the *Are* locus.

Selection efficiency and recombination frequency. Flanking markers have been shown to be more efficient in selection for a trait of interest (Tanksley, 1983). Combining marker data from 207 F_2 individuals belonging to both segregating populations, the bracketing RAPDs were able to select for the *Are* allele with 98.6% efficiency. Based on recombination frequency of the these two RAPD markers the theoretical selection fidelity reaches 99.7%. The benefit of bracketing markers for marker-assisted selection is not fully appreciated when individual markers are tightly linked to the gene of interest, which is the case of the present study. However with looser linkages, scoring for flanking markers would showed greater selection efficiency over single (closely linked) marker selection.

The introgression of the *Are* gene into the Andean gene pool from Middle American germplasm represents a wide cross. Haley et al., (1993), showed that genes

from the Middle American gene pool appear to exhibit tighter linkages with markers when measured in the Andean genetic background. A similar trend has been observed with Andean resistance genes when studied in Middle American segregating populations (Miklas et al., 1993). Lower recombination values between the *Are* allele and both RAPD markers were both expected and observed in the Andean mapping population. Although differences in recombination frequencies were not statistically supported, it is noteworthy that the two described RAPD markers were developed independently and both showed trends similar to previously reported observations. Recombination has specific mechanisms that are not clearly understood. Environmental effects may play a role in different recombination frequencies observed between populations. The present study was not designed to distinguish genotypic from environmental influences on estimates of recombination values. However the apparent difference in linkage between populations derived from the two gene pools might suggest that some form of recombinational suppression is occurring in wide crosses of common bean. A larger population size and/or a larger sample of crosses representing both gene pools might be needed to determine differences in recombination distance between germplasm pools. One property of wide crosses is the incorporation of genetic material that may not be sufficiently similar to permit the same degree of recombination as seen with crosses of more similar genetic material. Paterson et al., (1990) observed a diminished reciprocal exchange, between heterozygous chromosomal segments flanked by homozygous regions, in wide crosses between *Lycopersicon* species. They pointed out, that the lower likelihood of reciprocal exchange in a heterozygous chromosomal segment may be accentuated by positive

interference, from more frequent reciprocal exchanges in flanking homozygous regions. Any difference in the recombination value between gene pools of *P. vulgaris* might suggest the need to conduct mapping studies in populations in which breeders are actively working. Developing genetic maps using wide crosses may allow the mapping of a number of traits but may be of limited value to applied plant breeders interested in using markers to introgress an agronomically useful trait. Until linkages are generated and tested among related materials, map-based breeding will probably be restricted to the introgression of foreign genes.

Application of RAPD markers. The occurrence of two new races of *C. lindemuthianum* in North America has put in jeopardy the long-term strategy of relying on single gene resistance for controlling bean anthracnose (Kelly et al., 1994). Race 73 and alpha-Brazil overcome the resistance conferred by the *Are* gene, and race 7 is virulent on genotypes carrying the *A* gene. Since the *A* gene conditions resistance to races 73 and alpha-Brazil, *Phaseolus* breeders now need to pyramid the *Are* and *A* genes into new cultivars as the only effective means of conferring resistance to all current North American races of *C. lindemuthianum*. With the characterization of additional anthracnose resistance genes (Pastor-Corrales et al., 1994), MAS offers bean breeders a viable alternative for developing cultivars with pyramided resistance genes as a means of increasing the longevity of anthracnose resistance in common bean. As new broadly-based epistatic anthracnose resistance genes are being introgressed into commercial bean cultivars, using more virulent races of the pathogen, the $OQ4_{1440}$ and $B355_{1000}$ RAPD markers will be used to ensure the retention of the hypostatic *Are* gene in these materials. Since selection for the hypostatic *Are* gene will be dependent on linked markers in the

absence of test crosses and multiple anthracnose inoculations, flanking markers offer a significantly increased level of efficiency over single closely linked markers for marker based selection. Thus, long term_durable anthracnose resistance in common bean could be provided through the pyramiding of resistance genes using these RAPD markers.

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Chapter Three

RAPD MARKERS FOR MAJOR ANTHRACNOSE RESISTANCE GENES IN COMMON BEAN

ABSTRACT.

Pyramiding three major anthracnose resistance genes using marker-assisted selection (MAS) is now feasible in common bean. Four RAPD markers linked to the *A*, *Are 1024* and *Mexique 3* loci were developed using bulk segregant analysis and heterogeneous inbred population approaches. RAPD marker, $OF10_{530}$ was tightly linked in repulsion-phase to the *A* allele (1.9 ± 1.4 cM). A survey of bean genotypes for the presence or absence of the marker locus, revealed that $OF10_{530}$ RAPD would facilitate the introgression of the *A* gene across *Phaseolus* gene pools. A coupling-phase RAPD marker $OAH1_{780}$ and a repulsion-phase $OAK20_{890}$ RAPD marker, cosegregated with alleles at the *Are 1024* locus in three different segregating populations. The $OAH1_{780}$ and $OAK20_{890}$ RAPD markers were found linked (12.3 ± 1.9 and 7.3 ± 1.5 cM, respectively) and flanked the *Are 1024* locus. Coupling and repulsion-phase RAPD markers when used as a codominant pair showed higher selection efficiency, for the identification of homozygous (*Are 1024 Are 1024*) F_2 individuals, as opposed to individual selection for a coupling-phase or against a repulsion-phase RAPD phenotype. The RAPD marker $OAB3_{450}$ cosegregated with the *Mexique 3* allele in two different populations. The $OAB3_{450}$ RAPD was found linked at 5.9 ± 1.7 in coupling-phase with the *Mexique 3* allele. Pyramiding major anthracnose resistance genes using MAS is proposed, and the

effective durability of the resistance to *C. lindemuthianum* could be increased if Andean and Middle American genes were combined.

INTRODUCTION.

Colletotrichum lindemuthianum (Sacc. & Magnus) Lambs.-Scrib., the causal agent of anthracnose in common bean (*Phaseolus vulgaris* L.), is considered one of the most economically important fungal pathogens of the crop. The presence of the disease has been reported in most parts of the world where beans are produced and when favorable conditions for the establishment and growth of the pathogen exist (Pastor-Corrales and Tu, 1989). The main control measure against bean anthracnose has been genetic resistance. However, the extensive physiological variability exhibited by *C. lindemuthianum* has continuously lead bean breeders and pathologists to seek alternative genetic sources for long-term durability of the resistance (Beebe and Pastor-Corrales, 1991).

Gene pyramiding has been suggested as a strategy for stable resistance against variable plant pathogens (Nelson, 1978). Incorporating more than one gene into a single genotype is time-consuming and a difficult task due to epistatic interactions between resistance genes requiring extensive test crossing with different races of the pathogen, while, ensuring that the genotype possesses several other agronomic traits. Often, progeny testing might require screening with a new race of the pathogen to ensure the presence of a gene during the breeding process. Working with introduced isolates demands careful manipulation of the pathogen to avoid escapes and usually testing of genotypes is carried out under greenhouse conditions where space is limited.

Molecular markers offer the opportunity to pyramid major genes into a cultivar, since they are able to overcome the constraints usually encountered by conventional

breeding procedures (Tanksley, 1983; Melchinger, 1990; Stuber, 1991). The use of random amplified polymorphic DNA (RAPD), a polymerase chain reaction (PCR) based marker (Welsh and McClelland, 1990; Williams et al., 1990), can facilitate the transfer of a gene(s) when direct selection of a RAPD, closely linked to the gene of interest, is carried out. Marker assisted selection (MAS) using RAPD markers has been suggested as a potential useful breeding tool for gene introgression in common bean. At present, MAS is being implemented to pyramid major resistance genes affording complete protection to bean common mosaic virus (BCMV) and bean rust [*Uromyces appendiculatus* (Pers. ex Pers.) Unger var *appendiculatus*] (Kelly, 1995a).

The *Are* gene (Mastenbroek, 1960) is the first major anthracnose resistance gene for which molecular markers have been developed in common bean (Adam-Blondon et al., 1994; Young and Kelly, 1994). The RAPD marker, described by Young and Kelly (1994), linked in coupling to *Are* allele, could be used in MAS breeding to facilitate introgression of the *Are* gene across *Phaseolus* gene pools. In contrast, the utility in different genetic backgrounds, of the RAPD marker developed by Adam-Blondon et al. (1994) remains unknown, since, this RAPD was mapped in a population of Andean origin and no other germplasm screening was reported. Limited usefulness for MAS have been observed in other studies in common bean (Miklas et al., 1993; Haley et al., 1993; Johnson et al., 1995), where RAPD markers linked to disease resistance genes were found to be gene pool specific.

The *Are* gene has provided long-term effective protection against bean anthracnose in North America (Tu, 1992; Kelly et al., 1994a) and Europe (Fouilloux, 1979). However, the greater pathogenic variability of the fungus observed in Latin America has limited the

usefulness of this gene for anthracnose resistance (Menezes and Dianese, 1988; Rava et al., 1993). In the U.S., the introgression of the *A* gene, of Andean origin (McRostie, 1919) and the *Are* gene, of Middle American origin, into commercial cultivars, has now been overcome in North America. Recently, Kelly et al. (1994a) reported the presence in Michigan of two new races of *C. lindemuthianum*, races 7 and 73. Race 7 was virulent on cultivars carrying the *A* gene but was non-pathogenic on genotypes carrying the *Are* gene. Conversely, race 73 was virulent on cultivars carrying the *Are* gene and non-pathogenic on genotypes carrying the *A* gene. Concurrently, Tu (1994) reported the appearance of race alpha-Brazil which was also capable of defeating the genetic resistance conferred by the *Are* gene in Canada.

Two genotypes, AB 136 and G 2333, members of the anthracnose differential series, have shown broad resistance to a large collection of isolates from Latin America (Schwartz et al., 1982; Pastor-Corrales and Tu, 1989; Pastor-Corrales et al., 1995), including races 7, 73 and alpha-Brazil. It was observed that a single dominant gene was responsible for the resistance in AB 136 (Chapter 1), while, independent duplicate factors accounted for the resistance in G 2333 (Pastor-Corrales et al., 1994). A breeding line, SEL 1360, derived from G 2333, was found to carry one of two dominant genes present in the resistant parent (chapter 1). The two characterized dominant genes designated *Are 1024* (AB 136) and *Mexique 3* (SEL 1360), have been incorporated into the bean breeding lines at Michigan State University (MSU) as the new sources of resistance to anthracnose.

In the present study, research to find RAPD markers linked to three major anthracnose resistance genes, *A, Are 1024 and Mexique 3*, was conducted. The

implementation of molecular markers in MAS for gene pyramiding, seeking durable resistance to anthracnose in common bean, is discussed.

MATERIALS AND METHODS.

Genetic material. The MSU breeding line N85006 heterogeneous for the *A* locus, was the source of two near-isogenic lines (NILs), N85006 S (homozygous recessive for the *A* locus) and N85006 R (homozygous dominant for the *A* locus), which were assayed to identify linked RAPD markers. The N85006 is an $F_{2:6}$ line derived from the cross of MSU breeding lines N81058/N80038. The original source of the dominant *A* resistance gene in N85006 traces back to the anthracnose resistance in the cultivar Sanilac (Andersen et al., 1960). The development of NILs from advanced heterogeneous lines was described by Haley et al., (1994b); and the effectiveness of this strategy in molecular tagging of the *Are* resistance gene is discussed in Chapter 2. Progeny of 104 F_2 (104 $F_{2:3}$) individual plants from the N85006 S/N85006 R cross were used as the mapping population for the *A* locus.

The cultivar Catrachita, an small red bean type from Honduras, was used as the source of the *Are 1024* resistance gene (Beebe and Pastor-Corrales, 1991). Three different mapping populations (Black Magic/Catrachita, Raven/Catrachita and Blackhawk/Catrachita) with an average of 107 F_2 individual plants and their respective $F_{2:3}$ families, each, were used to confirm putative linkages between a RAPD marker and alleles at the *Are 1024* locus.

The source of the *Mexique 3* resistance gene was a CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia) breeding line (SEL 1360) derived from the differential cultivar G 2333 (Voysest et al., 1995). Testing of putative genetic linkages between RAPD markers and alleles at the *Mexique 3* locus was performed in two different mapping populations (Black Magic/SEL 1360 and Blackhawk/SEL 1360) averaging 103 F_2 individual plants with their respective $F_{2:3}$ families. The bulked segregant analysis approach (Michelmore et al., 1991) was used to form two contrasting DNA pairs (resistant and susceptible bulks) when screening for markers linked to the *Are 1024* and *Mexique 3* genes. The contrasting DNA bulks were composed of equal volumes of standarized DNA from six F_2 homozygous resistant and six F_2 homozygous susceptible individual plants. The F_2 individual plants, used to form the DNA bulks were derived from crosses between Black Magic/Catrachita and Raven/Catrachita segregating for the *Are 1024* gene and Black Magic/SEL 1360 and Blackhawk/SEL 1360 segregating for the *Mexique 3* gene. The parental material used in the formation of mapping populations and DNA bulks for *Are 1024* and *Mexique 3* genes was described in Chapter 1.

Disease evaluation. The genotype of the parental material, used as a source of DNA for RAPD screening, for each locus under study was first confirmed by inoculation with the appropriate race of the fungus. Alpha served as the discriminating race between N85006 S and N85006 R parental lines, in the segregating F_2 population and for progeny testing the $F_{2:3}$ families. Three different races of *C. lindemuthianum* were used for genotyping parental, F_2 individual plants and $F_{2:3}$ families, from crosses involving the *Are 1024* and *Mexique 3* genes. Race alpha was used for Black Magic/Catrachita and Black

Magic/SEL 1360 populations; race delta for Raven/Catrachita population; and race 73 for Blackhawk/Catrachita and Blackhawk/SEL 1360 populations. All progeny testing was conducted by spray inoculating 12 to 18 (10 to 12 day-old seedlings) $F_{2:3}$ individuals from each F_2 plant. The inoculation procedure and disease rating followed the description provided in materials and methods in Chapter 1.

RAPD analysis. Similar protocols for DNA extraction, PCR reactions for DNA amplification and cycling profile and visualization of PCR amplified products, used to tag the *Are* gene were used in the current study (see chapter 2). For resolution enhancement of a small polymorphic band generated by the primer OAB3, electrophoresis was performed in 2.7 % agarose gel, containing 0.5 ug ml^{-1} ethidium bromide, in 0.5X TBE buffer (0.045M Tris-borate and 0.001M EDTA). Primer amplifying products that exhibited polymorphisms between contrasting NILs and/or DNA bulks were subsequently evaluated in their respective segregating population. Simple inheritance of the disease phenotype and the putative linked RAPD markers were confirmed using Chi-square tests. Linkage analysis were performed using the program Linkage-1 (Suiter et al., 1983) and MAPMAKER (Lander et al., 1987), version 3.0. A logarithm of the odds ratio (LOD) score of 3.0 was established for linkage, and the Kosambi mapping function was used to calculate map distance in centimorgans (cM.).

RESULTS.

A total of 572 random decamer primers were used to screen the NILs heterogeneous for the *A* locus (N85006 S and N85006 R). Three putative RAPDs, two in repulsion ($OF10_{530}$ and $OAM4_{670}$) and one in coupling-phase ($OAL7_{530}$) with the *A* allele,

were polymorphic between the NIL pair. However, only one of these markers, $OF10_{530}$ (generated by a 5'-GGAAGCTTGG-3' decamer; Fig. 3.1), was found tightly linked with the *A* locus, at a distance of 1.9 ± 1.4 cM (Table 3.1). Dominant and monogenic inheritance of the anthracnose resistance phenotype and the $OF10_{530}$ RAPD is shown in Table 3.1. The presence of the *A* locus and the $OF10_{530}$ RAPD marker was surveyed in a series of common bean genotypes belonging to different market classes from different gene pools (Table 3.2). The $OF10_{530}$ RAPD appeared to be a gene pool non-specific marker, since, it was able to determine the genotype of the *A* locus in individuals regardless of genetic origin. In Sanilac, the source of the *A* gene in the NILs used for primer screening, the $OF10_{530}$ marker locus was absent; indirectly confirming the presence of the *A* gene in this cultivar. In a few individuals, however, the $OF10_{530}$ marker locus was either present (Seafarer) or absent (Isabella, G 2333, Perry Marrow, Kaboon), contrary to the expected genotype of the *A* locus.

A total of 216 decamer primers were used to screen the four contrasting DNA bulks for the *Are 1024* and the *Mexique 3* genes. Three putative RAPD markers were found to be linked to the alleles of interest. Polymorphisms between either contrasting DNA bulk was first confirmed using a second DNA bulk, prepared with individuals from a different F_2 population (segregating for the same gene of interest). All polymorphic band(s) which were consistent in both DNA bulks were also found cosegregating in the F_2 population used to construct both bulks. Confirming a putative linkage, by using a second contrasting DNA bulk, before the actual mapping of a RAPD in a segregating population, helped accelerate the process of primer screening, since false positives were readiliy discarded saving time and resources.

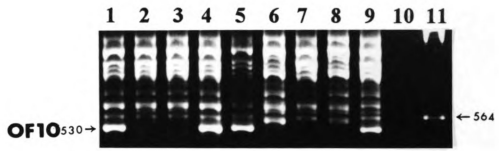

Figure 3.1 Amplification of genomic DNA using $OF10_{530}$ RAPD marker. Key to individuals: (1) anthracnose-susceptible NIL N85006-S, (2) anthracnose-resistant NIL N85006-R, (3) homozygous resistant F_2 individual, (4) heterozygous resistant F_2 individual, (5) homozygous susceptible F_2 individual, (6) Andean cultivar with *A* gene (Michigan Dark Red Kidney), (7) Middle American cultivar with *A* gene (Sanilac), (8) Andean cultivar with *A* gene (Montcalm), (9) Middle American cultivar without *A* gene (Cornell 49242), (10) negative control (no template DNA added to PCR amplification), (11) molecular weight marker (λ *Hind* III/*Eco*RI: size of bands indicated in bp)

Table 3.1 Chi-square (X^2) analysis for $OF10_{530}$ RAPD marker and the *A* allele segregating in the N85006 S/N85006 R F_2 and $F_{2:3}$ population.

Locus tested	Generation	Expected ratio	Observed frequency	X^2	Probability
A	F_2	3:1	75:29	0.32	0.57
A	$F_{2:3}$	1:2:1	30:45:29	1.90	0.39
$OF10_{530}$	F_2	3:1	76:28	0.12	0.73
Linkage estimate[z]					
a/ $OF10_{530}$	$X^2 = 94.5$	$P = 0.00$	1.9 ± 1.4 cM		

[z] Two point Chi-square (X^2) analysis and linkage estimate (3:6:3:1:2:1) based on 1:2:1 genotypic segregation ratio for the *A* locus and 3:1 ratio for the $OF10_{530}$ repulsion-phase RAPD marker.

Table 3.2 Survey of common bean genotypes from different market classes and gene pools for the presence of the *A* locus and the OF10$_{530}$ RAPD marker.

Genotype	*A*[z]	OF10$_{525}$[y]	Market class	GP[x]	Genotype	*A*[z]	OF10$_{530}$[y]	Markert class	GP[x]
MDRK	+	–	Kidney	A	SEL 1308	–	+	Black	MA
Chinook	+	–	Kidney	A	CN 49242	–	+	Black	MA
Montcalm	+	–	Kidney	A	Talamanca	–	+	Black	MA
Redkloud	+	–	Kidney	A	Pinto 114	–	+	Pinto	MA
Isabella	ND	–	Kidney	A	Aztec	–	+	Pinto	MA
Sanilac	+	–	Navy	MA	Fiesta	–	+	Pinto	MA
Bunsi	–	+	Navy	MA	Sierra	–	+	Pinto	MA
OAC Rico	–	+	Navy	MA	Alpine	–	+	Great Northern	MA
Seafarer	+	+	Navy	MA	Catrachita	–	+	Small red	MA
Raven	+	–	Black	MA	AB 136	–	+	Small red	MA
Blackhawk	–	+	Black	MA	G2333	ND	–	Small red	MA
Black Magic	–	+	Black	MA	Perry Marrow	ND	–	Marrow	A
SEL 1360	–	+	Black	MA	Kaboon	ND	–	Snap bean	A

[z] + = *A* allele present; – = A allele absent; ND = not determined. [y] + = OF10$_{530}$ present; – = OF10$_{530}$ absent.
[x] Gene pool: A = Andean, MA = Middle American; Singh et al. (1991).

population used to construct both bulks. Confirming a putative linkage, by using a second contrasting DNA bulk, before the actual mapping of a RAPD in a segregating population, helped accelerate the process of primer screening, since false positives were readiliy discarded saving time and resources

Two RAPD markers were found linked with alleles at the *Are 1024* locus. The RAPD marker $OAH1_{780}$ (generated by a 5'-TCCGCAACCA-3' decamer primer) was linked in coupling, whereas the RAPD marker Oak20890 (amplified by decamer 5'-TGATGGCGTC-3'), was linked in repulsion with the *Are 1024* resistance allele (Fig. 3.2 and Fig. 3.3). Segregation ratios, in three different populations, consistent with simple monogenic inheritance were observed for the marker loci and the *Are 1024* gene (Table 3.3). Linkage analysis showed a consistent linkage between the *Are 1024* gene and $OAH1_{780}$ in three different populations (Table 3.4). The $OAH1_{780}$ RAPD mapped at 14.0 ± 3.6, 10.7 ± 3.1 and 12.2 ± 3.3 cM from the *Are 1024* resistance allele in the Black Magic/Catrachita, Raven/Catrachita and Blackhawk/Catrachita populations, respectively. No significant differences in recombination values, calculated using the standard errors of the estimates, were observed among the three F_2 mapping populations. The repulsion-phase marker $OAK20_{890}$ was found linked to the *are 1024* allele, at a distance between loci of 6.5 ± 2.5 cM in the Black Magic/Catrachita, 12.5 ± 3.3 cM in the Raven/Catrachita and 3.0 ± 1.7 cM in the Blackhawk/Catrachita F_2 populations (Table 3.4). Difference in recombination frequency ($P = 0.01$), however, was observed between the Raven/Catrachita and the Blackhawk/Catrachita mapping populations. Recombination values between the two marker loci, observed among the three mapping populations were statistically not significant. The map distance between RAPD markers ranged from 21.6 ±

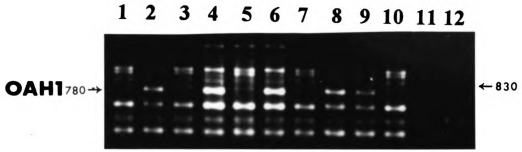

Figure 3.2 Amplification of genomic DNA using $OAH1_{780}$ RAPD marker. Key to individuals: (1) anthracnose-susceptible Black Magic/Catrachita DNA bulk, (2) anthracnose-resistant Black Magic/Catrachita DNA bulk, (3) susceptible parent, Black Magic, (4) resistant parent, Catrachita, (5) susceptible parent, Raven, (6) resistant cultivar, AB 136, (7) susceptible parent, Blackhawk, (8) homozygous resistant F_2 individual, (9) heterozygous resistant F_2 individual,(10) homozygous susceptible F_2 individual, (11) negative control (no template DNA added to PCR amplification), (12) molecular weight marker (λ *Hind* III/*Eco*RI: size of bands indicated in bp).

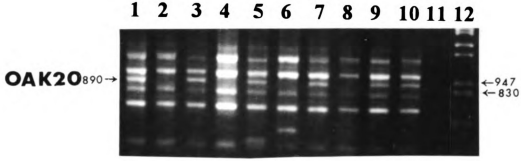

Figure 3.3 Amplification of genomic DNA using $OAK20_{890}$ RAPD marker. Key to individuals: (1) anthracnose-susceptible Black Magic/Catrachita DNA bulk, (2) anthracnose-resistant Black Magic/Catrachita DNA bulk, (3) susceptible parent, Black Magic, (4) resistant parent, Catrachita, (5) susceptible parent, Raven, (6) resistant cultivar, AB 136, (7) susceptible parent, Blackhawk, (8) homozygous resistant F_2 individual, (9) heterozygous resistant F_2 individual,(10) homozygous susceptible F_2 individual, (11) negative control (no template DNA added to PCR amplification), (12) molecular weight marker (λ *Hind* III/*Eco*RI: size of bands indicated in bp).

Table 3.3 Chi-square (X^2) analysis for $OAH1_{780}$ and $OAK20_{890}$ RAPD markers and the *Are 1024* allele segregating in three different F_2 and $F_{2:3}$ populations.

Population	Locus tested	Generation	Expected ratio	Observed frequency	X^2	Probability
Black Magic/Catrachita	*Are 1024*	F_2	3:1	74:32	1.26	0.26
Black Magic/Catrachita	*Are 1024*	$F_{2:3}$	1:2:1	23:50:32	1.78	0.41
Raven/Catrachita	*Are 1024*	F_2	3:1	76:34	1.75	0.19
Raven/Catrachita	*Are 1024*	$F_{2:3}$	1:2:1	28:47:34	2.73	0.26
Blackhawk/Catrachita	*Are 1024*	F_2	3:1	79:32	0.68	0.41
Blackhawk/Catrachita	*Are 1024*	$F_{2:3}$	1:2:1	23:55:31	1.18	0.55
Black Magic/Catrachita	$OAH1_{780}$	F_2	3:1	74:31	0.92	0.34
Raven/Catrachita	$OAH1_{780}$	F_2	3:1	75:34	1.91	0.17
Blackhawk/Catrachita	$OAH1_{780}$	F_2	3:1	74:35	2.57	0.11
Black Magic/Catrachita	$OAK20_{890}$	F_2	3:1	85:20	1.68	0.20
Raven/Catrachita	$OAK20_{890}$	F_2	3:1	86:23	0.69	0.41
Blackhawk/Catrachita	$OAK20_{890}$	F_2	3:1	87:22	1.10	0.29

Table 3.4 Two point Chi-square (X^2) analysis and linkage estimates for marker loci (OAH1$_{780}$ and OAK20$_{890}$) and the *Are 1024* locus.

Population[z]	Locus tested	Expected ratio[y]	Observed frequency	X^2	Probability	cM (r ± SE)
Black Magic/Catrachita	*Are 1024*/OAH1$_{780}$	3:6:3:1:2:1	21:46:7:2:4:25	52.3	0.00	14.0 ± 3.6
Raven/Catrachita	*Are 1024*/OAH1$_{780}$	3:6:3:1:2:1	28:41:6:0:6:28	61.6	0.00	10.7 ± 3.1
Blackhawk/Catrachita	*Are 1024*/OAH1$_{780}$	3:6:3:1:2:1	23:46:5:0:9:26	55.2	0.00	12.2 ± 3.3
Black Magic/Catrachita	*are 1024*/OAK20$_{890}$	3:6:3:1:2:1	4:50:31:19:0:1	77.3	0.00	6.5 ± 2.5
Raven/Catrachita	*are 1024*/OAK20$_{890}$	3:6:3:1:2:1	8:45:33:20:2:1	57.4	0.00	12.5 ± 3.3
Blackhawk/Catrachita	*are 1024*/OAK20$_{890}$	3:6:3:1:2:1	2:54:31:21:1:0	91.6	0.00	3.0 ± 1.7
Black Magic/Catrachita	OAH1$_{782}$/OAK20$_{890}$	9:3:3:1	55:19:30:1	7.1	0.01	21.6 ± 4.6
Raven/Catrachita	OAH1$_{782}$/OAK20$_{890}$	9:3:3:1	53:22:33:1	9.8	0.00	18.9 ± 4.3
Blackhawk/Catrachita	OAH1$_{782}$/OAK20$_{890}$	9:3:3:1	52:22:35:0	13.0	0.00	15.2 ± 3.8
Across populations[x]	*Are 1024*/OAH1$_{780}$	3:6:3:1:2:1	72:133:18:2:19:79	167.5	0.00	12.3 ± 1.9
Across populations[x]	*are 1024*/OAK20$_{890}$	3:6:3:1:2:1	14:149:95:60:3:2	221.9	0.00	7.3 ± 1.5
Across populations[x]	OAH1$_{780}$/OAK20$_{890}$	9:3:3:1	160:63:98:2	29.6	0.00	15.8 ± 2.3

[z] Anthracnose evaluation conducted on $F_{2:3}$ progenies. [y] Linkage analysis based on 1:2:1 genotypic segregation ratio for the *Are 1024* locus and 3:1 ratio for the OAH1$_{780}$ and OAK20$_{890}$ RAPD markers. [x] Includes data from Black Magic/Catrachita, Raven/Catrachita, and Blackhawk/Catrachita populations.

4.6 cM in the Black Magic/Catrachita, 18.9 ± 4.3 cM in the Raven/Catrachita and 15.2 ± 3.8 cM in the Blackhawk/Catrachita F_2 population (Table 3.4). Linkage estimates calculated across mapping populations (323 F_2 individuals) showed that the $OAH1_{780}$ RAPD was located at 12.3 ± 1.9 cM from the *Are 1024* allele, $OAK20_{890}$ RAPD mapped at 7.3 ± 1.5 cM from the *are 1024* allele and 15.8 ± 2.3 cM separated both RAPD marker loci (Table 3.4). Using the data across populations MAPMAKER analysis showed that the RAPD markers were flanking the *Are 1024* locus.

The third RAPD marker, designated $OAB3_{450}$ (generated by a 5'-TGGCGCACAC-3' decamer), identified using bulked segregant analysis, was found linked in coupling with the *Mexique 3* allele (Fig. 3.4). Monogenic inheritance of the *Mexique 3* gene was confirmed in two segregating populations (Black Magic/SEL 1360 and Blackhawk/SEL 1360), where the $OAB3_{450}$ marker segregated as a single factor (Table 3.5). Cosegregation analysis revealed that $OAB3_{450}$ RAPD was linked to the *Mexique 3* allele, at a distance of 7.2 ± 2.6 cM in the Black Magic/SEL 1360 population; and at 4.4 ± 2.1 cM in the Blackhawk/SEL 1360 population (Table 3.6). Combining data from all F_2 individuals (206) in both populations, the linkage estimate for $OAB3_{450}$ RAPD marker and the *Mexique 3* allele was 5.9 ± 1.7 cM (Table 3.6). No difference in recombination value among mapping populations was observed between the marker locus and the resistance allele.

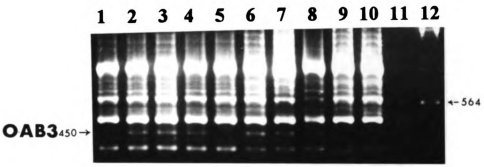

Figure 3.4 Amplification of genomic DNA using $OAB3_{450}$ RAPD marker. Key to Individuals: (1) anthracnose-susceptible Raven/SEL 1360 DNA bulk, (2) anthracnose-resistant Raven/SEL 1360 DNA bulk, (3) homozygous resistant F_2 individual,(4) heterozygous resistant F_2 individual, (5) homozygous susceptible F_2 individual, (6) resistant parent with *Mexique 3* gene, SEL 1360, (7) resistant cultivar, with *Mexique 3* gene, TU, (8) resistant cultivar with *Mexique 3* gene, G 2333, (9) resistant line without *Mexique 3* gene, SEL 1308, (10) resistant cultivar without *M exique 3* gene, AB 136, (11) negative control (no template DNA added to PCR amplification), (12) molecular weight marker (λ *Hind* III/*Eco*RI: size of bands indicated in bp).

Table 3.5 Chi-square (X^2) for the *Mexique 3* allele and the $OAB3_{450}$ RAPD marker segregating in two different F_2 and $F_{2:3}$ populations.

Population	Locus tested	Generation	Expected ratio	Observed frequency	X^2	Probability
Black Magic/SEL 1360	*Mexique 3*	F_2	3:1	83:27	0.00	1.00
Black Magic/ SEL 1360	*Mexique 3*	$F_{2:3}$	1:2:1	29:54:27	0.11	0.95
Blackhawk/SEL 1360	*Mexique 3*	F_2	3:1	83:28	0.00	1.00
Blackhawk/SEL 1360	*Mexique 3*	$F_{2:3}$	1:2:1	33:48:28	2.01	0.41
Black Magic/SEL 1360	$OAB3_{450}$	F_2	3:1	76:33	1.35	0.25
Blackhawk/SEL 1360	$OAB3_{450}$	F_2	3:1	74:23	0.03	0.86

Table 3.6 Two point Chi-square (X^2) analysis and linkage estimates for $OAB3_{450}$ RAPD marker and the *Mexique 3* allele.

Population[z]	Observed frequency[y]	X^2	Probability	cM (r ± SE)
Black Magic/SEL 1360	28:47:1:1:5:27	78.5	0.00	7.2 ± 2.6
Blackhawk/SEL 1360	28:43:3:0:1:22	77.0	0.00	4.4 ± 2.1
Across populations[x]	56:90:4:1:6:49	153.9	0.00	5.9 ± 1.7

[z] Anthracnose evaluation conducted on $F_{2:3}$ progenies. [y] Linkage analysis, expected ratio = 3:6:3:1:2:1, based on 1:2:1 genotypic segregation ratio for the *Mexique 3* allele and 3:1 ratio for the $OAB3_{450}$ RAPD marker. [x] Includes data from Black Magic/Sel 1360 and Blackhawk/SEL 1360 populations.

DISCUSSION.

Based on our data, bean breeders have the opportunity to pyramid up to four major anthracnose resistance genes (*A, Are 1024* and *Mexique 3*, this Chapter and; *Are*, Chapter 2) using RAPD markers for MAS. The combination of specific resistance genes would afford complete protection against all existing races of *C. lindemuthianum* in North America, and in regions of Latin America where higher pathogenic variability has been observed. The value of the *A, Are 1024 and Mexique 3* major genes and the application of the linked RAPD markers in breeding for anthracnose resistance using MAS is now possible.

A gene. The Andean *A* gene was the first major gene utilized to develop anthracnose resistant cultivars in common bean (Burkholder, 1918; McRostie, 1919). With the discovery of the Middle American *Are* gene by Mastenbroek (1960), which confers resistance to six races of *C. lindemuthianum* (Tu, 1992), the utilization of the *A* gene as the main genetic source for anthracnose resistance began to decline. However, the *A* gene regained importance after Kelly et al. (1994a), reported the appearance of two new races of anthracnose in Michigan, and proposed pyramiding *A* and *Are* genes as a suitable breeding strategy for controlling the emerging pathogenic variability. The combination of Andean and Middle American resistance genes seems to be a sound strategy for the control of bean anthracnose. For instance, it was observed, that several highly virulent isolates of *C. lindemuthianum* pathogenic on resistance genes of Middle American origin, were avirulent on the Andean *A* gene (Pastor-Corrales et al., 1994); conversely, isolates of Andean origin have been observed avirulent on Middle American

resistance genes (Pastor-Corrales et. al., 1995). Hence, the *A* gene could be a valuable source for anthracnose resistance in regions where most of the contemporary cultivars in use are derived from Middle American germplasm, e.g. Mexico, Central America and Brazil (Rava et al., 1993; Voysest et al., 1994). The tightly linked (1.9 ± 1.4 cM) repulsion-phase $OF10_{530}$ RAPD marker can be used in MAS, selecting against the presence of the RAPD, to identifiy homozygous dominant individuals for the *A* locus. It is also noteworthy that the OF10 primer was found polymorphic and linked in coupling with a major rust resistance (B-190) gene block in common bean (Haley et. al., 1993). Although the OF10 RAPD marker had limited application within the Middle American gene pool for selection of the B-190 gene, it offers the unique opportunity for simultaneously introgressing two major resistance genes (*A* and B-190) into the great northern and pinto bean market classes. There seemed to be no restriction for the implementation of $OF10_{530}$ RAPD for introgressing the *A* gene across gene pools. However, in the cultivar Seafarer, which is known to carry the *A* gene (see appendix), the marker locus was present. The $OF10_{530}$ RAPD marker was absent in Isabella, G 2333, Perry Marrow and Kaboon cultivars, but there is insufficient genetic information regarding the presence or absence of the *A* gene in these genotypes. However, more than one genetic factor conditions resistance to the alpha race in Isabella (see appendix), G 2333 (Pastor-Corrales et al., 1994), Perry Marrow (Del-Peloso et al., 1989; Gonçalves-Vidigal, 1994) and Kaboon (Muhalet et al., 1981) and the marker data suggests that the *A* gene could be one of them.

***Are 1024* gene.** The anthracnose differential cultivar AB 136, a small red bean type of Middle American origin, was the original source of the *Are 1024* gene. The

genetic resistance conferred by *Are 1024* has proven to be effective to a large number of Latin American isolates of *C. lindemuthianum*, and is an important resistance source used by breeding programs in Central America (Schwartz et al., 1982; Pastor-Corrales and Tu, 1989). Finding two RAPD markers in opposite orientation and flanking the *Are 1024* allele provides the opportunity to compare different selection strategies based on the phenotype of individual markers or the combination of both as a codominant pair. A comparison of selection efficiency for homozygous resistant individuals, using actual observations, by means of MAS using a coupling-phase, repulsion-phase and/or a codominant marker is shown in Table 3.7. Using a codominant marker, scoring individuals simultaneously with both coupling ($OAH1_{780}$) and repulsion ($OAK20_{890}$) RAPD markers, a level of 95% selection efficiency was observed, while 92% efficiency was obtained by selecting against the $OAK20_{890}$ repulsion-phase marker alone. The increased effectiveness of genotype selection observed using RAPDs as codominant markers versus repulsion marker-based selection, was due to the ability of the codominant marker as opposed to the repulsion-phase marker to distinguish, thus discard, double recombinant individuals. Recognition of double recombinants by RAPD markers used as a codominant pair has been cited as the main reason for the increased level of selection efficiency over a repulsion-phase marker in MAS (Johnson et al., 1995). Tight linkages ($\leq$ 5 cM) between markers and genes of interest is required for efficient marker-based selection (Tanksley, 1983). Linkages greater than five centimorgans, however, can efficiently be used in MAS providing markers flanking the locus of interest are available.

Table 3.7 Selection efficiency (%) observed based on selection for a coupling RAPD $OAH1_{780}$ and against a repulsion RAPD $OAK20_{890}$, and for both RAPD markers scored as one codominant marker ($OAH1_{780}$- $OAK20_{890}$) in MAS for homozygous resistant individuals for the *Are 1024* resistance gene segregating in three different F_2 populations.

Genotypic frequency	No. of recombinants (C:R)[z]	Coupling $OAH1_{780}$	Repulsion $OAK20_{890}$	Codominant $OAH1_{780}$-$OAK20_{890}$
RR-74	2:4	32	92	95
Rr-152	19:3	59	5	5
rr-97	18:2	8	3	0
No of selected F_2 individuals		226	65	63

[z] Number of recombinant individuals observed using C = coupling-phase $OAH1_{780}$ RAPD marker and R = repulsion-phase $OAK20_{890}$ RAPD marker.

This fact was confirmed with the $OAH1_{780}$ and $OAK20_{890}$ RAPDs which were considered loosely linked markers (12.3 ± 1.9 and 7.3 ± 1.5 cM, respectively). Since $OAH1_{780}$ and $OAK20_{890}$ RAPDs flanked the *Are 1024* locus and could be combined as a codominant marker, higher levels of selection efficiency over selection based on single coupling or single repulsion-phase RAPD phenotype were possible (Table 3.7). Coupling-phase RAPD linkages are usually sought during primer screening for molecular tagging. Selection against a repulsion-phase RAPD marker, however, resulted in an increased selection efficiency of homozygous resistant individuals (92%) as opposed to 33% selection efficiency for the coupling-phase RAPD in a F_2 population segregating for the *Are 1024* resistance gene (Table 3.7). Greater selection efficiency of repulsion-phase over

coupling-phase RAPD markers for MAS has been demonstrated in common bean for BCMV resistance (Haley et al., 1994b) and bean rust resistance (Johnson et al., 1995).

Estimates of recombination frequency for the $OAK20_{890}$/*Are 1024* loci between the Raven/Catrachita and Blackhawk/Catrachita mapping populations, were statistically different. Factors affecting the rate of recombinational events among plants of a particular population, may be genetically or environmentally influenced (Brown et al., 1989; Beavis and Grant, 1991). Although the segregating populations are genetically related and were developed during the same season and under the same environmental conditions, the present research was not designed to discriminate between such factors which alter recombination. The development of three different segregating populations for estimating genetic linkages, helped to increase the level of accuracy when determining map distance between loci of interest. Concurrently, testing cosegregation of RAPD markers and resistance loci over different genetic backgrounds served to test the potential usefulness of the marker, for future MAS application, in genetically diverse breeding populations.

Mexique 3 gene. The genetic characterization of the Middle American *Mexique 3* gene, present in the differential cultivar TU, was reported by Fouilloux (1979) in Europe where it has been used extensively as a source of anthracnose resistance. It was shown, that the *Mexique 3* gene was present in SEL 1360, a black bean, derived from the differential cultivar G 2333 (Chapter 1). The $OAB3_{450}$ RAPD marker found linked in coupling-phase to the *Mexique 3* allele (5.9 ± 1.7 cM) served to confirm the presence of the gene in TU, SEL 1360 and G 2333 and its absence in the other anthracnose differential genotypes (data not shown). In Chapter 1 we proposed that G 2333 may in fact carry three factors conditioning resistance to bean anthracnose, rather than the two proposed by

Pastor-Corrales et al., (1994). Since the previous authors used race 521 to screen their segregating population, the *Mexique 3* gene would have not been detected because it is overcome by race 521. We have clearly demonstrated in genetic (Chapter 1) and DNA analysis (marker) that G 2333 carries the *Mexique 3* gene. Since G 2333 possesses the most effective race-specific resistance to *C. lindemuthianum* currently observed, finding markers linked to the other resistance genes will be complicated by the presence of more than one segregating factor. Research to separate and tag these resistance factors in G 2333 is underway, while the $OAB3_{450}$ RAPD marker will be used to assist in the introgression and pyramiding of the *Mexique 3* gene into commercial bean cultivars.

Gene pyramiding. The importance of incorporating a diverse group of resistance sources and/or combining Andean and Middle American genes for stable resistance to bean anthracnose has been suggested (Pastor-Corrales and Tu, 1989; Beebe and Pastor-Corrales, 1991; Kelly et al., 1994a; Pastor-Corrales et al., 1995). Major resistance genes such as *A, Are, Are 1024 and Mexique 3*, which have shown broad-based resistance, could be pyramided into a single genotype, since, RAPD markers linked to alleles at these loci can now be used in MAS breeding. If gene pyramiding is aiming to increase the longevity of anthracnose resistance in common bean, then the number of genes really needed to attained durable resistance becomes important. Based on the resistance to stripe rust (*Puccinia striiformis* West. var. *striiformis*) observed in several winter wheat cultivars grown in Western Europe during more than 15 years, Parlevliet (1993), suggested that the cause of the lasting resistance was due to the presence of three fully effective race-specific genes pyramided into single cultivars. Similarly, Schafer and Roelfs (1985), indicated that the increase in durability of resistance to wheat stem rust (*Puccinia graminis* Pers. f. sp.

tritici) in the northern plains was associated with multiple race-specific genes (6) pyramided into a single genotype. Durability of the resistance was related to the low probability of mutation to virulence occurring at various loci in the pathogen population. Other potential mechanisms suggested responsible for the durability of resistance gene pyramids are associated with: the reduced fitness of a pathogen race which has accumulated a large number of virulence genes (Van Der Plank, 1975; Green and Campbell, 1979); the difficulty of combining multiple virulence mutations with high pathogen fitness; and the chance discovery of single resistance genes or combinations of small numbers of resistance genes that are more durable than average (Mundt, 1990, 1991). It would appear that breeding for gene pyramids will result in the increase of durability of resistance, regardless of the mechanism involved. However, there is no clear correlation between the number of resistance genes and the durability of the resistance (Mundt, 1991) which suggests that some gene combinations might be more effective than others. A large number of resistance genes deployed singly in a cultivar or genetic factors other than race-specific genes, might be involved in increasing durable resistance (Johnson, 1981).

We propose combining two genes into different genotypes and cultivar deployment of such genetic combinations, would be more practical and realistic to efficiently provide long-term control of bean anthracnose at a reasonable cost and in a relatively short period of time. Genetic diversity of resistance sources, suggested as a means of expanding host resistance and increased durability, would be attained if the Andean *A* gene is combined with any of the other three Middle American genes (*Are, Are 1024* and *Mexique 3*) in different combinations. Ideally, when breeding for a particular region, the breeder should

carefully select a gene pair that if deployed singly, would confer resistance to all known races in that region. For example, in the U.S. and Canada the latter can be accomplished if *Are 1024* and *Mexique 3* genes are selected for gene pyramiding. However for other areas a suitable genetic pair and correspondence molecular markers might not always be available. For instance, *Are, Are 1024 and Mexique 3 genes* as opposed to the *A* gene, may have limited use in some areas of Central America due to presence of virulent races of the pathogen that overcome the resistance conferred by these three Middle American genes individually (Rava et al., 1993). For those situations, it would be advisable to choose genes with a comprehensive race coverage and complementary to each other. Thus, pyramiding *A* with any of the Middle American genes as a complementary gene pair would be the best strategy to follow for complete protection to bean anthracnose in that region. A similar complementary genetic pair could also be generated for the control of the disease in North America. Two commercial bean cultivars (Isles and Newport) with the gene combination (*A* and *Are*) have already been released in Michigan (Kelly et al.,1994b, 1995b); so the durability of the resistance in these cultivars remains to be determined. The usefulness and application of MAS as a breeding tool for gene pyramiding directed towards durable disease resistance will differ between breeding programs. In some instances, the presence of a gene combination could be detected using discriminating races of the pathogen alone, or require the use of a linked molecular marker to select for a hypostatic gene combined with race inoculation to ensure the selection of the epistatic gene in segregating populations. Plant breeders will need to assess when to

use MAS as a strategy based on cost, time, space, and the availability of races to effectively discriminate between resistance genes masked by epistasis.

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APPENDIX

APPENDIX

Is the Anthracnose Resistance '*A*' Gene the Same in Cultivars Belonging to Both Bean Gene Pools?

The *A* gene was one of the first anthracnose resistance genes reported in the literature (McRostie, 1919). Since its discovery, this single dominant gene, which conditions resistance to the alpha race of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.- Scrib, has been incorporated into cultivars from both gene pools. Currently, it is not clear if resistance to the alpha race observed in contemporary Mesoamerican and Andean cultivars, traces back to a single origin.

One of the first bean cultivars known to carry a resistance gene effective against the alpha race was Wells' Red Kidney (Barrus, 1915). Crosses between this kidney bean and selections of Michigan Robust (a white navy bean), were carried out by McRostie to introduce anthracnose resistance into the navy bean. Based on this evidence, one might speculate that the alpha resistance gene in Andean and Mesoamerican cultivars has a single origin in Wells' Red Kidney. Genetic resistance to the alpha race in Emerson 847, a navy bean breeding line, and Dark Red Kidney was shown to be governed by a dominant gene at the *A* locus (Cardenas et al., 1964). This raises two important questions: is the source of the *A* gene the same for cultivars from both gene pools? and is there only a single dominant gene that conditions resistance to the alpha race? Certainly, many of the current commercial cultivars have either Emerson 847 or Dark Red Kidney in their pedigree. For

instance, Sanilac and Seafarer (navy bean cultivars), derives their resistance to alpha from Emerson 847 (Andersen et al., 1960); whereas Montcalm (a kidney bean cultivar), derives its resistance to alpha from Michigan Dark Red Kidney (Copeland and Erdmann, 1977).

In order to determine if the same alpha resistance gene is present in navy and kidney bean cultivars, the following cross was made: Montcalm x Seafarer. Parental material, a susceptible check and 103 F_2 individual plants derived from this cross were inoculated with a spore suspension of the alpha race. Apart from symptoms on the susceptible check, none of the segregating F_2 plants and neither parental genotype showed any anthracnose symptoms. These results suggest that both Seafarer and Montcalm carry identical genes corresponding to the *A* locus.

In order to verify if other kidney bean cultivars carried the same *A* gene, a second cross between Isabella x Seafarer was made. Isabella, a light red kidney bean, was released by MSU as an alpha resistance cultivar (Kelly et al., 1987), but no known anthracnose resistance parent is recognized in its pedigree ('Redkloud/Mecosta'). The disease evaluation of the F_2 population of the Isabella x Seafarer cross generated a different outcome. Among the 177 F_2 individual plants tested, 21 plants were rated as susceptible (disease symptoms present), whereas 156 plants were rated as resistant (disease symptoms absent) to the alpha race. Chi-square tests performed on this data supported a 57:7 ratio ($P = 0.78$), suggesting the segregation of three factors (3:1, *A* gene in Seafarer; 9:7, duplicate complementary factors in Isabella). The data clearly shows that a different genetic system conditions resistance to the alpha race in Isabella. Progeny tests performed in the F_3 generation confirmed the F_2 observations. The source of the

resistance to alpha in Isabella is unknown, and, as demonstrated, is not controlled by the *A* gene.

The finding that Isabella carries different alpha resistance factors, was recently confirmed by Kelly et al. (1994), using two new anthracnose races reported in Michigan. They found that one of these races (Race 73), in addition to defeating the *Are* resistance gene, was also pathogenic on Isabella. However, Race 73 was non pathogenic on Seafarer, Montcalm, Sanilac and Michigan Dark Red Kidney cultivars, which all carry the *A* gene.

For years, anthracnose resistance in North America has relied on the protection conferred by the *Are* gene. The appearance of race 73 has put the usefulness of the *Are* gene in jeopardy, whereas the *A* gene has regained importance. Hence, pyramiding *Are* and *A* genes is a sound breeding strategy for developing anthracnose resistant bean cultivars where variability in *C. lindemuthianum* is a potential threat.

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