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DISSECTION OF R-GENE MEDIATED ANTHRACNOSE RESISTANCE IN *PHASEOLUS VULGARIS*

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DISSECTION OF R-GENE MEDIATED ANTHRACNOSE RESISTANCE IN *PHASEOLUS VULGARIS*

By

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ABSTRACT

DISSECTION OF R-GENE MEDIATED ANTHRACNOSE RESISTANCE IN *PHASEOLUS VULGARIS*

By

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Anthracnose, caused by the fungal pathogen *Colletrotrichum lindemuthianum*, is one of the most economically important diseases of common bean (*Phaseolus vulgaris*) worldwide. A comprehensive molecular characterization of genomic and transcriptional factors which contribute to resistance is necessary to facilitate breeding for anthracnose resistance. In this study two anthracnose resistance genes from the Andean and Middle American bean gene pools were characterized using molecular marker and traditional genetic techniques. In addition, expression analysis was used to identify genes which are constitutively expressed or induced during the incompatible interaction that may contribute to the resistance.

An amplified fragment length polymorphism (AFLP) marker ($E_{ACT}M_{CCA}$-108) was identified, linked at 9.9 cM from the Andean resistance locus, *Co-1*^{2}. The AFLP marker was converted to an STS marker ($SE_{ACT}M_{CCA}$) and mapped to linkage group B1 of the integrated bean linkage map. Additionally, it was determined that the Andean cultivar Jalo EEP558 carries the *Co-1* locus using molecular markers, genetic characterization and inoculation with various races. The *Co-7* anthracnose resistance

locus, originally from the Middle American landrace G 2333, was isolated in a breeding line named MSU7. The resistance spectrum of the *Co-7* gene was determined and an AFLP marker linked to the gene at 4.9 cM was identified.

Several constitutively expressed genes involved in primary metabolism were identified that have a probable role in the ability of the bean plant to mount *Co-4*^{2} mediated resistance. Additionally, several genes, differentially regulated between inoculated and mock-inoculated treated plants in the incompatible interaction, were identified. Changes in gene expression were detected as early as 1 hour post inoculation. Among the genes identified was a putative serine/threonine receptor kinase, a putative lysine decarboxylase-like enzyme and a NIM1-like protein. The products of these genes have previously been correlated with the defense response in a number of other taxa. I speculate that the combination of constitutive expression differences in primary metabolism processes and genes involved in active defense contribute to resistance mediated by the *Co-4*^{2} gene in common bean.

DEDICATION

To my family, who has sacrificed so much to get me to where I am today, and to my husband, Justin, for his unconditional love and encouragement.

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GENERAL INTRODUCTION

Anthracnose. *Colletotrichum lindemuthianum*, the causal fungus of bean anthracnose, has global distribution affecting not only common bean but also cowpea (*Vigna uniguiculata*) and tepary bean (*P. acutifolius* var. *latifolius*) among other less economically important species of *Phaseolus* and *Vigna* (Tu, 1982). Anthracnose is the most economically important disease of common bean worldwide (Melotto et al., 2000 b). Yield losses of up to 95% have been reported (Guzman et al., 1979). The two most important environmental components which affect infection by *C. lindemuthianum* and the expression of disease symptoms are temperature and humidity (Pastor-Corrales and Tu, 1989). The conidia are produced in acervuli, and are encased in a hydrophilic mucilage called the spore matrix (Bailey et al., 1983). The optimum temperature range for conidia development is 18 to 20°C. A relative humidity of at least 92% and moisture on the foliage for 12 hours post conidia deposition are important for germination (Tu, 1982). The symptoms usually appear on the cotyledonary leaves as small, dark brown to black lesions. Later, the lesions may appear on veinlets on the abaxial surface of the leaves, and on the petiole and larger leaf veins. Lesions can also appear on stems and lead to plant girdling and death. The pathogen often invades the pod, and mycelia and conidia can infect the cotyledons or seed coat of the developing seeds where it can lie dormant. Pod infection appears as flesh to rust-colored lesions that develop into sunken cankers with a slightly raised black ring border. Severely infected young pods are often aborted. The infected seed may also contain dark brown to black cankers making them not suitable for marketing (Pastor-Corrales & Tu, 1989). Sporulation that occurs within lesions produces secondary inoculum. *C. lindemuthianum* spores are splash-dispersed

over short distances and wind-dispersed over longer distances (Tu, 1992). Seedlings grown from infected seed often result in damping off as their hypocotyls tend to break off from diseased areas (Tu, 1982). In addition to the highly efficient transmission of anthracnose through seed, another problem which can lead to an outbreak is that the fungus can survive in seed or infected crop residues for many years under low moisture conditions in the form of conidia or sclerotia. The overall result of disease is yield loss, marketability of seed and seed quality (Tu, 1982).

Control strategies. Chemical, cultural and genetic control strategies are available for bean anthracnose with varying effectiveness. Spraying with fungicides at flower initiation, late flowering and pod fill, however, has had limited success in controlling anthracnose (Pastor-Corrales and Tu, 1989). In addition, the high cost of repeated chemical application makes this control strategy impossible for growers in developing countries, where farming is primarily subsistence and beans are a critical component of the diet (Broughton et al., 2003). The efficacy of seed treatment is also marginal due to variable location of the pathogen within the seed. Cultural controls, such as the use of anthracnose-free seed and crop rotation are highly recommended. Anthracnose-free seed can be produced in environmental conditions, such as in a hot and semi-arid climate, which are unfavorable for the development of the pathogen. Two or three year crop rotation is suggested because of the ability of the pathogen to survive in crop debris (Lenné, 1992). In developing countries the production and distribution of anthracnose-free seed is not generally feasible, therefore, the use of resistant cultivars remains the most practical control strategy. The extensive use of resistant cultivars, anthracnose-free

seed and crop rotation has considerably reduced the importance of anthracnose in North America, Europe and Australia (Pastor-Corrales and Tu, 1989).

C. lindemuthianum. *Colletotrichum lindemuthianum* is an ascomycete and is the causal agent of bean anthracnose. *C. lindemuthianum* has a perfect and imperfect stage. Because the telomorph is very rare in nature, and even in culture, the fungus is known by its anamorph name, *Colletotrichum lindemuthianum.* The telomorph was first identified as *Glomerella cingulata* f.sp. phaseoli but was then classified by Shear as *Glomerella lindemuthianum.* Since the imperfect stage is very rare in nature, anthracnose is primarily caused by the anamorph, therefore, the most common disease cycle is described in Figure 1. Briefly, conidia, produced in acervuli, that have over-wintered on plant debris or infected seed, germinate and infect the plant which leads to the development of symptoms. Secondary inoculum is produced in the infected areas of the plant and is spread by wind, rain, animals or equipment.

C. lindemuthianum is a hemibiotrophic fungus, that is, it has a biotrophic and a necrotrophic growth phase. Infection is established through a brief biotrophic phase, associated with large intracellular primary hyphae. Later, the fungus switches to the necrotrophic phase which is associated with narrower secondary hyphae which ramify throughout the host tissue (TeBeest et al., 1997). During the biotrophic phase, the host plasma membrane invaginates, surrounding the infection vesicle and primary hyphae. An interfacial matrix is deposited at the host/pathogen interface. Twenty-four hours post penetration, functional integrity of the host plasma membrane is lost and the host cell begins to degrade and die. Cell death is confined to infected cells at this point and not

associated with extensive host cell wall dissolution. No macroscopic symptoms are observed since dead tissue does not become brown. As the primary hyphae colonize new cells, biotrophy is established in each newly infected cell, thus biotrophy and necrotrophy may occur simultaneously. The appearance of secondary hyphae announce the start of the highly destructive necrotrophic phase. The secondary hyphae are narrow and move both inter- and intracellularly. During this stage, host cells are rapidly killed in advance of fungal growth. Host cells are extensively degraded and the typical symptoms of anthracnose become visible (TeBeest et al., 1997).

Pathogen variability. *C. lindemuthianum* has worldwide distribution, having been reported on all continents (Pastor-Corrales and Tu, 1989). The classification of *C. lindemuthianum* isolates into races was first made by Barrus (1911). Due to the rare occurrence of sexual recombination and the belief that dispersal over great distances was limited, initially, pathogenic variability was thought to be low (Beebe and Pastor-Corrales, 1991). Pathogen variability was difficult to assess because of the lack of a universal system of race classification. Currently, virulence variability in *C. lindemuthianum* is assessed based on the reaction of a given isolate on a standard differential series of 12 common bean genotypes and a binary system based on the position of each cultivar within this series (Pastor-Corrales, 1991). The adoptation of this standard procedure permits the comparison of data from various research groups. Throughout this dissertation I will sometimes refer to *C. lindemuthianum* "strains" and other times "races". This is an unfortunate consequence of some researchers not adopting the universal nomenclature used to characterize races.

The combination of virulence and molecular analyses of *C. lindemuthianum* populations has expanded the breadth of understanding of variability within and between fungal populations. Intraspecific diversity has been analyzed using ribosomal DNA polymorphism (Balardin et al., 1999). To assess intraspecific diversity among 57 isolates, the authors used PCR primers specific to the rDNA region comprising the two internal transcribed spacers (ITS1 and ITS2) and the 5•8S rRNA gene in *C. lindemuthianum*, and digested the resulting amplicons with endonucleases (PCR-RFLP analysis). The isolates tested divided into two groups based on the PCR-RFLP analysis. Although Group I consisted mainly of Middle American races (65%), and group II consisted mainly of Andean races (85%), neighbor-joining and parsimony analyses of the sequence data did not support an association of any particular ITS genotype with host gene pool, virulence or geographic origin of races, suggesting that virulence can arise in different geographic regions at different times, independent of genetic background (Balardin et al., 1999). Intra-race polymorphisms were observed among isolates of races 7, 17, 31 and 73 collected in various countries using PCR-RFLP of the ITS regions. These results support a greater level of molecular variability within *C. lindemuthianum* than that determined by virulence analysis alone and suggests that specific virulence patterns evolved independently. More recently, Mahuku and Riascos (2004) assessed genetic variability of 200 *C. lindemuthianum* isolates collected from Andean and Middle American varieties and regions using virulence on the differential series, DNA sequence of repetitive-elements (Rep-PCR) and random amplified microsatellites (RAMS). Pathotypic (90 pathotypes identified) and genetic (0.97) diversity among the 200 isolates revealed a high level of diversity. This diversity, however, does not cluster by bean gene

pools and the authors suggest that the high diversity observed in the Middle American region indicates that *C. lindemuthianum* originated from this region.

Host resistance. There are three general categories of the host response to infection with *C. lindemuthianum*: extreme resistance, extreme susceptibility and intermediate. Extreme susceptibility is characterized by the development of fungal infection vesicles within living epidermal cells, followed by further colonization of host cells by intracellular primary hyphae and a transient biotrophic relationship is re-established in each newly colonized cell. Six to seven days post inoculation, brown, water-soaked lesions appear and the fungus switches to necrotrophic growth, that results in the breakdown of plant cell walls and the death of plant cells in advance of fungal growth (O'Connell and Bailey, 1988). Extreme resistance is characterized by the rapid and localized death of epidermal cells soon after penetration. Infection vesicles are not formed and the growth of the fungus is restricted to the penetrated cell (O'Connell et al., 1985). The gene-for-gene (GFG) relationship between *C. lindemuthianum* and *P. vulgaris* was proposed as expressed as either the survival of the initially infected epidermal cell or its rapid death (Bailey, 1983), although, most studies involving the inheritance of anthracnose resistance evaluate host response at the plant level rather than at the single cell level. The intermediate category of host response to *C. lindemuthianum* was created to accommodate other less extreme host responses. This category ranges from small groups of dead cells, which appear as flecks, to large areas of necrosis, classified as limited lesions. Plants which are placed in the intermediate response

category, often survive and yield well, and consequently are considered to be resistant by some research groups and partially resistant by others.

Resistance to bean anthracnose, primarily conditioned by major genes (*Co-1* through *Co-10*), is believed to function in a GFG manner with pathogenicity in *C. lindemuthianum*, although some quantitative trait loci (QTL) for partial resistance have also been reported (Geffroy et al., 2000). The GFG theory, proposed by Flor (1947), describes when one gene in the plant corresponds with one gene in the pathogen to mediate the compatible/incompatible reaction. The gene in the plant which mediates this response is referred to as a resistance gene (R-gene). R-genes are inherited, most often, in a single dominant fashion, although, examples of recessively inherited resistance s not uncommon (Deslandes et al., 2002; Huang et al., 1997; Johansen et al., 2001). This GFG relationship between *P. vulgaris* and *C. lindemuthianum* is inferred since there have not been studies conducted on the inheritance of pathogenicity in the fungus due to problems with ascospore viability (Bryson et al, 1992). Each anthracnose resistance gene conditions resistance to numerous races of *C. lindemuthianum* and several genes (*Co-1*, *Co-3* and *Co-4*) are reported as complex loci with multiple alleles. This is in accordance with genetic studies that revealed that genes efficient against different strains of a specific pathogen were often located at complex loci displaying a multiallelic structure and/or a cluster of linked genes responsible for different specificities (Crute and Pink, 1996; Pryor and Ellis, 1993).

As an alternative to major genes which condition resistance to anthracnose, Geffroy et al. (2000) focused on the identification of QTL that contribute to anthracnose resistance. Geffroy et al. (2000) identified 10 putative QTL scattered across the genome

(Figure 2) contributing from 11-76% of the phenotypic variation. Some of the QTL are strain-specific and co-localize with other anthracnose resistance genes or defense response genes. In this study, the selection of *C. lindemuthianum* strains and the resistance source were critical to detecting QTL. This analysis was conducted using two strains of *C. lindemuthianum* that produce differential symptoms on the parental material where only partial resistance was observed. Tissue- and strain-specific QTL were detected. A critical assessment of this study is made difficult because the authors did not characterize their fungal strains by the universal anthracnose differential series.

Resistance genes. Gene-for-gene disease resistance in plants involves two basic processes: the perception of the pathogen and the response by the plant to limit disease. Based on the combination of structural motifs, most cloned R-genes fall into five classes (Jones, 2001) (Figure 3). Class 1 contains just one member, Pto (tomato), which contains a serine-threonine kinase (STK) catalytic domain. Additionaly, Pto has a N-terminal myristilation motif. The second class is made up of proteins which have leucine-rich repeats (LRRs), a putative nucleotide binding sit (NBS), and an N-terminal putative leucine-zipper (LZ) or other coiled-coil (CC) sequence. Class 3 R-gene proteins are the same as class 2 but instead of the CC sequence, these proteins have a TIR domain which is homologous to the N terminus of the Toll and Intertleukin 1 receptor (IL-1r) proteins. The notable lack of a transmembrane (TM) domain in proteins belonging to classes 1 through 3, suggests that these are be intracellular proteins (Figure 3). Class 4 R-genes lack an NBS and have a TM and an extracellular LRR. The fifth class of R-gene proteins

has a single member, Xa21 protein from rice. This protein has an extracellular LRR, a TM and a cytoplasmic STK domain (Jones, 2001).

No R-genes have been cloned from *P. vulgaris*, however, several R-gene-like sequences have been discovered. Melotto and Kelly (2001) identified a 1,110 bp open reading frame (ORF), closely linked to the *Co-4*^{2} anthracnose resistance gene. This ORF, named *COK-4*, encodes a STK domain, highly similar to the *Pto* gene in tomato, but with a highly hydrophobic membrane-spanning region. Melotto et al. (2004) cloned several additional copies of the *COK-4* gene from the same region, identifying a cluster of STK sequences. A family of related LRR sequences has been identified in the region of the *Co-2* anthracnose resistance gene (Geffroy et al., 1998). Three of these LRRs were further characterized and found to be members of the NBS-LRR class of R-gene proteins (Creusot et al., 1999). Ferrier-Cana et al. (2003) identified four NBS-LRR sequences which map to bean linkage group B4, co-localizing with previously identified QTL and major genes (*Co-y*, *Co-z*, and *Co-9*) for anthracnose resistance. Additionally, Rivkin et al. (1999) identified several NBS containing genes from *P. vulgaris* with conserved kinase domains. Some of these NBS sequences mapped near the *Ur-6* gene which confers resistance to *Uromyces appendiculatus*.

Most cloned R-gene proteins are grouped into either class 2 (NBS-LRR with N-terminal CC domain) or class 3 (NBS-LRR with N-terminal TIR domain) type proteins (Ellis et al., 2000). Class 1 is the only class of R-genes lacking an LRR, therefore, R-gene proteins appear to rely on a limited number of structural and functional domains, of which the LRR must be important. The LRR domain contains leucine or other hydrophobic residues at regular intervals (~24 amino acids in length) (Bent, 1996).

Leucine-rich repeat domains of proteins from yeast, Drosophila and humans are known to mediate protein-protein interactions (Bent, 1996). Because LRR were known to play a role in protein-protein interactions in eukaryotes, researchers speculated that the LRR may function in recognition of the pathogen via binding of an elicitor molecule in plants. Support for this functional role comes from the identification of mutant alleles of RPS2 (Bent et al., 1994; Mindrinos et al., 1994) and RPM1 (Grant et al., 1995), which are nonfunctional due to single amino acid changes within the encoded LRR region. In addition to recognition, some studies suggest roles for the LRR in signaling. For instance, point mutations in the LRR domain of the *Arabidopsis* RPS5 resistance protein partially suppresses multiple bacterial and downy mildew resistance genes, suggesting a dominant negative interaction with a shared signaling component (Warren et al., 1998).

The NBS domain of R-genes, sometimes referred to as a P-loop, is an amino acid motif which is present in a variety of proteins having ATP or GTP binding activity (Martin et al., 2003). The NBS is part of a larger domain that includes homology between R-gene proteins and eukaryotic cell death effectors, Apaf-1 and Ced4. Together, this larger domain is referred to as the NB-ARC or Ap-ATPase domain. Because the NBS domain is highly conserved in some R-genes, the functionality of these proteins is likely to be dependent on binding nucleotide triphosphate (Martin et al., 2003). The CC motif of R-gene proteins is a repeated heptad sequence which has hydrophobic amino acid residues. The LZ is an example of a CC structure (Martin et al., 2003). The LZ, found in many different proteins, is believed to promote the formation of CC structures facilitating protein-protein interactions and is better known for its role in the dimerization of eukaryotic transcription factors (Bent, 1996). In *Arabidopsis*, the R-gene proteins

which contain CC domains, depend on downstream signaling components that are different from those required by TIR-NBS-LRR proteins. This suggests that the CC domain may function in signaling rather than in recognition (Martin et al., 2003). Serine-threonine kinases are proteins involved in phosphorylation and thus are believed to be involved in signal transduction of the defense response (Hardie, 1999). Both Pto and the kinase domain of Xa21 are functional STKs (Martin et al., 2003).

Hypersensitive response. Stakman (1915) is generally credited as the first to use the term 'hypersensitive' to describe the rapid and localized plant cell death induced by rust fungi in rust-resistant cereals. The term 'hypersensitive response' was adopted when it was clear that such cell death was a common expression of disease resistance in plants, regardless of pathogen (Heath, 2000). This response is defined as a rapid, localized cell death at the site of pathogen infection (Kombrink and Schmelzer, 2001). Characteristic of the hypersensitive response (HR) is the presence of brown, dead cells at the site of infection, although, HR is not restricted to those cells which have been infiltrated by the pathogen (Heath, 2000).

Most R-genes trigger the HR, although, mutational studies indicate that the HR also depends on genes which are present in both resistant and susceptible genotypes, termed 'required for disease resistance genes' or RDR genes, even in non-GFG interactions. For instance, mutations in the *NDR1* (a putative membrane associated protein) gene of *Arabidopsis*, suppresses resistance which is mediated by class 2 but not class 3 R-genes, whereas, the opposite is the case with mutations in *EDS1* (a putative lipase). This suggests that there must be more than one pathway that leads to HR cell death. The activated pathway may be more dependent on the class of R gene rather than

the pathogen (Heath, 2000). In *A. thaliana*, mutations at the *DND1* locus cause plants to be defective in HR cell death, but retain characteristic responses to avirulent *Pseudomonas syringae* such as the production of pathogenesis-related proteins and strong restriction of pathogen development (Yu et al., 1998). In *P. vulgaris*, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase and chitinase transcripts accumulated after infiltration with a Hrp-mutant strain of *Pseudomonas syringe* pv *tabaci* in the absence of a HR. Transcript accumulation occurred in the same temporal pattern as seen with infiltration with a wild-type strain which elicited the HR (Jakobek and Lindgren, 1993). These and other studies in which HR cell death was uncoupled from the induction of defense genes and disease resistance (del Pozo and Lam, 1998; Heath, 1998), also suggest that there are unique biochemical events associated with the HR, distinct from other plant defenses.

Additional characteristic features of R-gene mediated resistance response are the induction of phytoalexin production, the synthesis of chitinases and glucanases and the reinforcement of the cell wall. Early events in the signal transduction pathway of the defense response include calcium ion flux, specific changes in protein phosphorylation, the generation of activated oxygen species and the production of salicylic acid (Bent, 1996).

Co-evolution of *P. vulgaris* and *C. lindemuthianum*. Wild beans are found from northern Mexico to the north-west region of Argentina (Gepts and Debouck, 1991). *P. vulgaris* was domesticated in two distinct regions, Middle America, primarily Mexico, and along the eastern slope of the Andes in South America, specifically southern Peru, Bolivia and northwestern Argentina (Gepts et al., 1986). A minor domestication center

has been suggested in Colombia, however, it is unclear whether this area represents a domestication center or a region of gene flow between wild and domesticated bean gene pools (Gepts and Bliss, 1986; Beebe et al., 1997). The divergence of the two major gene pools during domestication has been supported by many morphological and molecular studies: variability in seed size (Evans, 1973), variability in the major seed storage protein, phaseolin, (Gepts et al., 1986), isozyme (Koenig and Gepts, 1989; Singh et al., 1991) and DNA marker variability (Becerra-Velásquez and Gepts, 1994; Haley et al., 1994). In addition to the above mentioned gene pools, recently discovered wild populations, considered to be ancestral, constitute a third gene pool in the region of Ecuador and northern Peru (Debouck et al., 1993; Kami et al., 1995; Tohme et al., 1996). Based on their unique phaseolin type, which is absent from the domesticated gene pools, these ancestral populations are not believed to have been involved in the domestication of common bean (Debouck et al., 1993).

The two major gene pools belong to the same species, despite a partial reproductive isolation caused by complementary lethal genes (Koinange and Gepts, 1992) and thus provide unique opportunities for breeders. A notable example is that of Beaver and Kelly (1994) who employed recurrent selection strategies to improve the yield potential of large-seeded red beans. Another good example of utilizing the genetic differences between gene pools is in breeding for anthracnose resistance.

Anthracnose resistance genes are categorized according to the gene pool origin of the host cultivar. The races of *C. lindemuthianum* are also similarly classified depending on the gene pool of the host cultivar for which each race was isolated (Balardin & Kelly, 1998). Isolates from the Andean region have a narrow virulence range and are more

virulent on cultivars of Andean origin, whereas Middle American isolates have a broader virulence range and predominantly attack Middle American cultivars (Beebe & Pastor-Corrales, 1991; Pastor-Corrales et al., 1995; Cattan-Toupance et al., 1998; Geffroy et al., 1999). Several studies on various host/pathogen interaction have shown that plants are more resistant to allopatric pathogens than to sympatric ones (Parker, 1985; Nevo, 1986; Lawrence and Burdon, 1989). This is consistent with theoretical predictions for conditions when either migration rates are low for both host and pathogen or migration rate of the host is lower than that of the pathogen (Gandon et al., 1996).

If resistance to anthracnose is controlled via a GFG relationship between host and pathogen, this would imply that bean plants carry R-genes corresponding to Avr genes from *C. lindemuthianum* races which may have originated in the opposite gene pool. The inference is that bean plants must carry many R-genes which confer resistance to allopatric races. This raises the question: why would a plant maintain R-genes for races of a pathogen for which it is not exposed (also termed unnecessary R-genes)? Geffroy et al. (1999) presented several possible explanations: (1) the unneeded specificities might be maintained because of their linkage to other R-genes, (2) these apparently unneeded genes might provide a renewable resource for the generation of new R-genes by genetic re-assortment events, (3) these R-genes may have other roles in the biology of the plant and might thus be involved in its fitness, (4) these genes may function locally against other pathogens or races delaying epidemics, (5) these genes may be maintained because they force the pathogen to limit the number of virulence factors it can use. Although, this may suggest a co-evolution of host and pathogen leading to parallel gene pools, when one considers pathogen studies, the literature on the pathogenic variation of *C.*

lindemuthianum is conflicting. Several research groups have found evidence to support the co-evolution of host and pathogen (Pastor-Corrales, 1996; Sicard et al., 1997; Balardin and Kelly, 1998, Ansari et al., 2003) yet others have determined that there is no association between virulence phenotype and geographic region or host gene pool (Fabre et al., 1995; Balardin et al., 1997; Balardin et al., 1999; Mahuku and Riascos, 2004). The conflict in the literature may be a product of the use of bred genotypes by some researchers. Using genotypes which result from breeding programs, the identity and origin of the resistance genes are not known and might confound the classification of isolates into distinct groups congruent with the diversity in *P. vulgaris*. Another possible reason for the conflict is that the separation and classification of pathotypes is dependent on the differential series. The present differential series contains only four Andean genotypes, all of which carry the *Co-1* gene, and eight Middle American genotypes, some of which have multiple genes which condition resistance to anthracnose. To clarify the question of co-evolution of *C. lindemuthianum* and *P. vulgaris*, a thorough analysis based on a large set of Andean and Middle American wild genotypes and pathotypes is required before strong conclusions about co-evolution can be made. Geffroy et al. (1999) carried out a similar study to analyze the molecular evolution of R-genes and the host-pathogen co-evolution process at the population level. The authors did not, however, assess molecular diversity of the *C. lindemuthianum* strains used. They collected 48 wild *P. vulgaris* plants from the three centers of diversity of the host species (Middle American, South Andean and North Andean) and cross inoculated them with 26 strains of *C. lindemuthianum*. They found that most of the resistance specificities (R-genes) were overcome in sympatric situations, indicating an adaptation of the pathogen to the local

host. In contrast, plants were generally resistant to allopatric strains which suggests that R-genes that were efficient against exotic (allopatric) strains but had been overcome locally were maintained in the plant genome. The authors concluded that co-evolution between *P. vulgaris* and *C. lindemuthianum* has led to a differentiation for resistance in the three centers of diversity of the host. Additionally, they mapped phenotypic resistance and several resistance gene analogs (RGAs) and found a cluster comprising both Andean and Middle American resistance specificities, suggesting that this locus existed prior to the separation of the two major gene pools of *P. vulgaris*.

Comparative studies of wild and cultivated populations of *P. vulgaris* have shown greater genetic diversity in wild populations, suggesting a founder effect of domestication (Gepts, 1990; Sonnante et al., 1994). The domesticated Andean gene pool is diverse in plant and seed morphology as well as agroecological adaptation, however, based on molecular analyses has a narrow genetic base as compared to Middle American germplasm (Beebe et al., 2001). AFLP analysis revealed that wild Andean bean populations have undergone more geographic isolation from each other as compared to most Middle American wild beans. Therefore, less genetic "mixing" has resulted in discrete populations in southern Peru, Bolivia and northern Argentina (Tohme et al., 1996). Interestingly, only one Andean anthracnose resistance gene has been characterized (*Co-1*) and four putative Andean resistance genes have been identified (*Co-w*, *Co-x*, *Co-y* and *Co-z*) (Kelly and Vallejo, 2004). The low number of Andean anthracnose resistance genes identified, relative to the number of Middle American (*Co-2* through *Co-10*) genes, is not surprising considering that the Andean gene pool is lower in genetic diversity than the Middle American.

Breeding for anthracnose resistance. Despite the extensive literature on resistance to anthracnose, bean breeders still struggle with the decision as to which gene(s) to deploy in resistance breeding programs. Independence of the nine anthracnose resistance genes (*Co-3* is believed to be allelic to *Co-9*) distributed across the genome (Kelly and Vallejo, 2004) offers bean breeders the unique opportunity to pyramid complementary resistance genes (Duvick, 1996) and, in certain cases, based on it's resistance spectrum, to choose the most effective allele at different loci, as the most prudent strategy in breeding for durable resistance to anthracnose (Kelly and Miklas, 1998). When breeding for anthracnose resistance in a particular geographic area, careful consideration should be taken in choosing which genes to combine. Ideally, each gene of a pyramid should, if deployed singly, condition resistance to all known races in that specific geographic region (Kelly and Miklas, 1998). For example, the combination of the *Co-5* and *Co-6* anthracnose resistance genes would meet this criterion in North America (Young and Kelly, 1996). To expedite the process of gene pyramiding, a combination of marker-assisted selection, and confirmation of successful gene introgression by inoculation with select races, is the best strategy for anthracnose resistance breeding (Kelly et al., 2003). Given the recent activity in mapping and gene tagging, new information on the location of most major genes controlling resistance to anthracnose is now available (Kelly et al., 2003). The availability of markers linked to specific anthracnose resistance genes, and alleles, in the case of multi-allelic loci such as *Co-1*, *Co-3*, *Co-4* and *Co-9* provide essential tools for the construction of pyramids with genes of complementary resistance spectra. The identification of markers linked to

anthracnose resistance genes is particularly important when it is not possible to confirm introgression by inoculation with discriminating races. This is the case when one gene is hypostatic to the other and a discriminating race cannot be identified.

Although most anthracnose resistance genes are inherited independently of each other, they are also in some cases clustered with other resistance genes in the bean genome (Kelly et al., 2003). Gene clusters of anthracnose and *Ur*-rust resistance genes are located on linkage groups B1, B4 and B11 (Figure 2). The Andean resistance genes for anthracnose, *Co-1*, and rust, *Ur-9*, cluster on B1, whereas Middle American genes, *Co-3/Co-9*, *Ur-5*, and Andean genes *Co-y*, *Co-z* cluster on B4. Other Middle American genes, *Co-2* and *Ur-3/Ur-11*, cluster on B11 (Kelly et al., 2003).

In the case of anthracnose resistance genes, clusters have been reported at the *Co-2* and *Co-9* loci (Creusot et al., 1999; Ferrier-Cana et al., 2003; Geffroy et al., 1999; Mendez-Vigo et al., 2005). The breakdown of resistance conferred by *Co-2*, a gene that confers resistance to a wide array of races, may have been aggravated by the disruption of the resistance gene cluster. Similarly, resistance conferred by *Co-2* could have broken down due to recombination events within the locus thus disrupting the functionality of closely linked resistance genes (Kelly and Vallejo, 2004). Although multiple alleles at other anthracnose resistance loci have been reported, the possibility that there are multiple closely linked genes in a cluster is not excluded by the allelism test. Therefore, it is very likely that gene clusters do exist at loci where multiple alleles have been previously identified. Allelism studies on populations of limited size (n=100), detect only linkage between two loci being tested, therefore one can only conclude whether two loci are less than or greater than 50 cM apart. This test for independence does not detect

recombination events that occur within the putative cluster, because only one anthracnose race is typically used in the analysis. Despite the evidence of resistance gene clusters, practical breeding for resistance may not be greatly facilitated by this knowledge. The value of mapping for breeders is not the associated traits that might be carried along in crossing, but the knowledge of independence of resistance genes should facilitate the pyramiding of different genes to enhance resistance. Although prudent pyramiding of resistance genes and deployment of appropriate resistance gene combinations has proven to be an adequate method of anthracnose control in many parts of the world, the addition of QTL that confer partial resistance offers breeders additional tools in selecting for durable resistance particularly in areas where pathogen diversity is extremely high.

GENERAL OBJECTIVES

Colletotrichum lindemuthianum is a highly variable and widespread pathogen, which makes breeding for durable anthracnose resistance a serious challenge. Although many major anthracnose resistance genes have been characterized and quantitative resistance is being investigated, Andean sources of resistance remain under characterized and utilized. New sources of resistance, both Andean and Middle American must be characterized to provide breeders various options to exploit the best combination of genes for the release of locally resistant bean cultivars. Tools which facilitate the introgression of these resistance traits are required to expedite the selection process. In this dissertation, the objectives were to develop molecular markers linked to the Andean *Co-1*^{2} gene and characterize a new Andean source of resistance, to characterize the Middle American anthracnose resistance gene *Co-7* and lastly, to investigate the expression of genes involved in the early resistance response to *C. lindemuthianum*. The research project is divided into three major sections, each of which is represented by a chapter in this dissertation. The objectives in the first chapter were to develop a molecular marker linked to the *Co-1*^{2} gene from the highly resistant Andean cultivar Kaboon, and to characterize the anthracnose resistance present in the Andean cultivar Jalo EEP558, used as a parent in the bean integrated linkage map. The second chapter objectives were to develop bean lines which are homozygous for the *Co-7* gene, characterize the spectrum of resistance conditioned by the *Co-7* gene and find an amplified fragment length polymorphism (AFLP) marker linked to this locus. The third chapter objectives were to identify genes with a putative role in the defense response by investigating constitutive

differences between plants which carry the anthracnose resistance gene, *Co-4*^{2}, and plants which do not, and by investigating changes in gene expression during the incompatible interaction between *C. lindemuthianum* and *P. vulgaris* by the cDNA-AFLP technique.

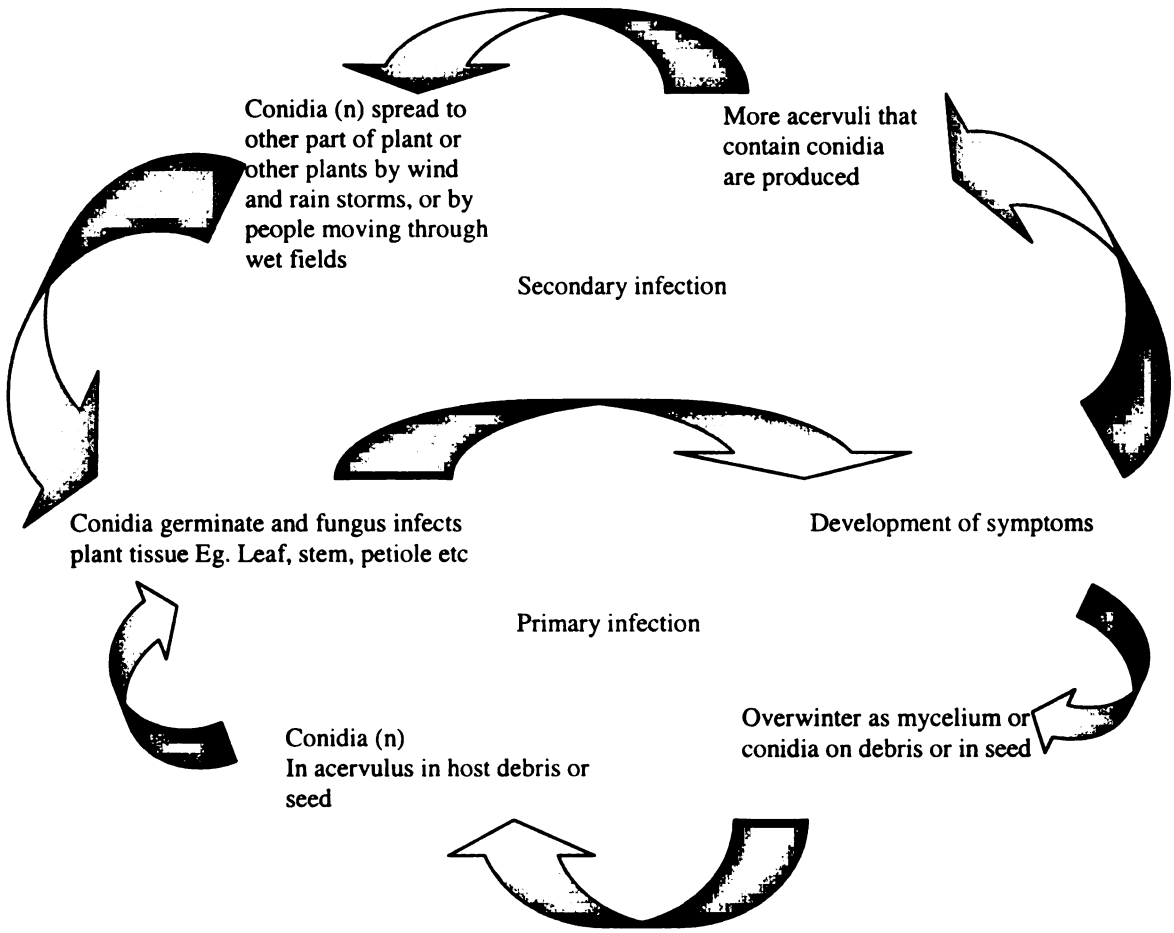

Figure 1. The disease cycle of anthracnose in *P. vulgaris*.

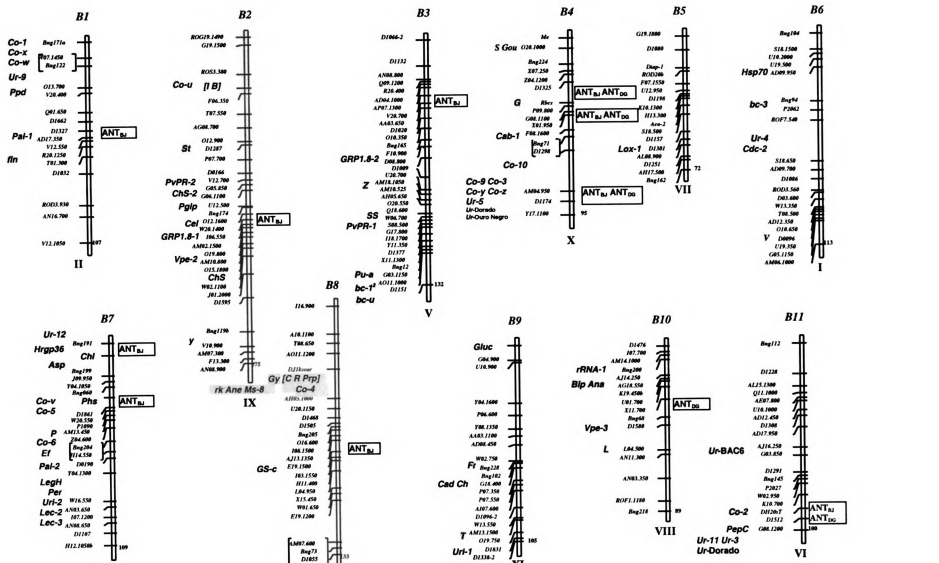
B1
Co-1
Co-x
Co-w
Ur-9
Ppd
Pal-1
fin
ANT_BJ
II
B2
Co-u
[I B]
PvPR-2
ChS-2
Pgip
Cel
GRP1.8-1
Vpe-2
St
ChS
ANT_BJ
IX
B3
Z
SS
PvPR-1
Pu-s
bc-1
bc-u
ANT_BJ
V
B4
S Gou
G
Cab-1
Co-10
Co-9 Co-3
Co-y Co-z
Ur-5
Ur-Dorado
Ur-Ouro Negro
ANT_BJ ANT_DG
ANT_BJ ANT_DG
ANT_BJ ANT_DG
X
B5
Lox-1
VII
B6
Hsp70
bc-3
Ur-4
Cdc-2
I
B7
Ur-12
Hrgp36
Asp
Co-v
Co-5
P
Co-6
Ef
Pal-2
LegH
Per
Uri-2
Lec-2
Lec-3
Chl
Phs
ANT_BJ
ANT_BJ
IV
B8
y
Gy [C R Prp]
Co-4
GS-c
ANT_BJ
III
B9
Gluc
Fr
Cad Ch
T
Uri-1
XI
B10
rRNA-1
Bip Ana
Vpe-3
L
ANT_DG
VIII
B11
Ur-BAC6
Co-2
PepC
Ur-11 Ur-3
Ur-Dorado
ANT_BJ
ANT_DG
VI

Figure 2. Common bean linkage map. Genomic distribution of genes with a biochemical function (mostly disease response genes), major genes coding for phenotypic traits, and QTL in common bean. This map is from Kelly and Vallejo (2004) with the following changes: *Co-5* was mapped to B7 by Campa et al. (2005) and *Co-3* and *Co-9* have been mapped to the same location on B4 (Méndez-Vigo et al., 2005). To the left of each linkage group, are the framework molecular markers (smaller font) and the biochemical genes (larger font) and major phenotypic trait genes (shaded boxes). The *Co* loci are anthracnose resistance loci. The *Ur* loci are rust resistance loci including the *Pu-a* for abaxial leaf pubescence. The *I* gene is a dominant resistance and *bc-u*, *bc-1*^{2} and *bc-3* are recessive genes for resistance to Bean Common Mosaic Virus. [*AI- Arl-Lec*] is the main locus coding for the -amylase inhibitor-arcelin-lectin multigene family. *Fin*, *Ppd*, *St* are genes for determinacy, sensitivity to photoperiod, and pod string formatio. *B*, *[C-R]*, *G*, *P*, and *rk* are seed color genes and *Ana*, *Ane*, *Bip*, *L*, *T* and *Z* are seed color pattern genes. *Asp* is a gene for seedcoat shininess. To the right (boxed symbols), are QTL for anthracnose (ANT) mapped in different populations. Location of most genes is approximate, as most were not directly mapped in the BAT93/Jalo EEP558 population from which this map is derived. The total distance of each linkage group (expressed in Kosambi cM) is shown at the right of the location of the bottom marker of that linkage group.

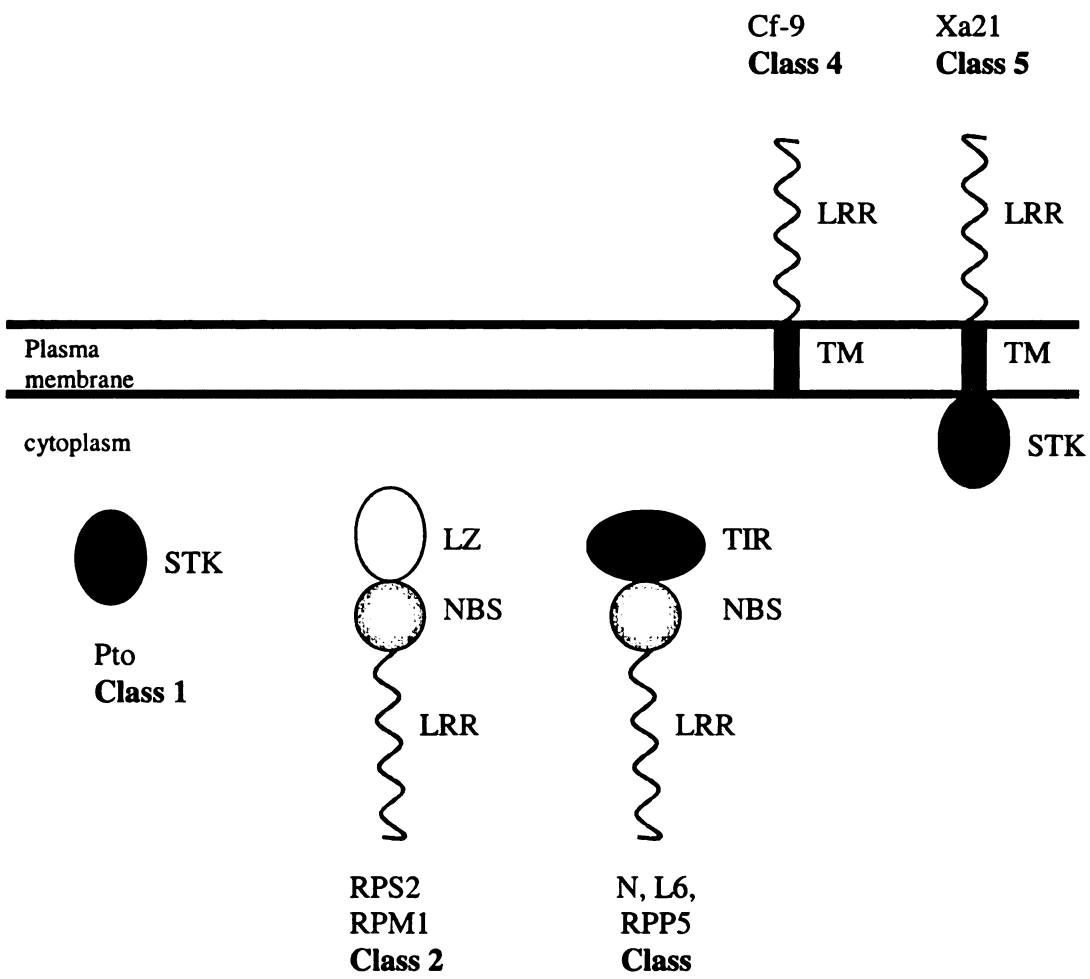

Figure 3. Pictoral representation of the 5 classes of R-genes. Xa21: *Oryza sativa* gene for *Xanthomonas oryzae*. Cf-9: *Lycopersicon esculentum* gene for *Cladosporium fulvum* resistance. Pto: *Lycopersicon pimpinellifolium* gene for *Pseudomonas syringae* resistance. N: *Nicotiana tabacum* gene for tobacco mosaic virus. RPS2, RPM1: *Arabidopsis thaliana* resistance to *Pseudomonas syringae*. L6: *Zea mays* gene for *Melampsora lini* resistance. RPP5: *Arabidopsis thaliana* gene for *Peronospora parasitica*.

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CHAPTER ONE

MOLECULAR TAGGING OF THE *CO-1²* LOCUS AND GENETIC CHARACTERIZATION OF THE ANTHRACNOSE RESISTANCE IN ANDEAN BEAN CULTIVAR JALO EEP558

ABSTRACT

The anthracnose resistance gene, *Co-1*, is a complex multiallelic locus, with 4 characterized alleles (*Co-1* - *Co-1⁴*) that has been previously mapped to bean linkage group B1 on the core bean (*Phaseolus vulgaris*) integrated linkage map. The objectives of this study were to develop a molecular marker linked to the *Co-1²* gene, from the Andean anthracnose differential cultivar Kaboon, and to characterize the anthracnose resistance in the Andean cultivar Jalo EEP558. Two strategies were employed to find a marker linked to the *Co-1²* locus (1) screen random AFLP primer pair combinations using bulked segregant analysis, (2) screen RAPD and SSR primers located on linkage group B1 for linkage to *Co-1*. The AFLP marker, $E_{ACT}M_{CCA}$-108/107 was identified, linked at 9.9 cM from the *Co-1²* allele. An STS marker, $SE_{ACT}M_{CCA}$, was derived from $E_{ACT}M_{CCA}$-108/107. This marker is not allele specific, but the STS and AFLP markers appear to be gene pool specific. The low level of polymorphism observed near the *Co-1* locus could be due to its close proximity to a block of highly conserved domestication genes. The anthracnose resistance in the Andean cultivar Jalo EEP558 was characterized using a combination of traditional and molecular techniques. Inoculation with various races of *C. lindemuthianum* revealed that Jalo EEP558 carried an allele of the *Co-1* locus, most likely the *Co-1* allele. The inoculation of the F_2 population, Jalo EEP558/Perry Marrow (*Co-1³*), with race 357, which yields an R/S (resistant/susceptible) reaction in the

parents, revealed that resistance to race 357 in Jalo EEP558 is conditioned either by one dominant and one recessive gene with epistasis or one dominant gene. The allelism test was conducted in the Jalo EEP558/MDRK F_2 population which was inoculated with race 73. This population of 200 individuals did not show segregation for resistance suggesting that resistance to race 73 is conditioned by an allele of the *Co-1* locus.

INTRODUCTION

Anthracnose, caused by the hemibiotrophic fungus *Colletotrichum lindemuthianum*, is considered the most economically important disease of common bean worldwide. This is due, in part, to the seed-borne nature and pathogenic variability of *C. lindemuthianum*. Genetic resistance is the best form of control, especially in areas of subsistence farming, commercial seed production and the certified seed industry. Pyramiding of anthracnose resistance genes of Andean and Middle American origin has been proposed as a strategy for broad-based durable resistance breeding (Young and Kelly, 1996a; Young and Kelly, 1996b). Many Middle American anthracnose resistance genes have been characterized, *Co-2* through *Co-10*, (Kelly and Vallejo, 2004), however, only *Co-1*, *Co-x* and *Co-y* are from Andean origin. New sources of resistance, therefore, are always being sought to provide genetic resources for anthracnose resistance breeding.

Co-1 is a complex locus, with 4 characterized alleles (Kelly and Vallejo, 2004), each conferring resistance to a different range of *C. lindemuthianum* races. The Andean differential cultivar, Kaboon, was shown to be highly resistant to anthracnose, only defeated by two of 40 races tested (Balardin et al, 1997). The major dominant gene conditioning resistance in Kaboon is reported to be $Co\text{-}1^2$, an allele of the *Co-1* gene (Melotto and Kelly, 2000). The Andean bean cultivar, Jalo EEP558, used as a parent in the mapping population of the bean integrated map, also demonstrates resistance to anthracnose (Freyre et al., 1998; Geffroy et al., 1999) and may provide a new source of resistance in the Andean gene pool.

Molecular markers linked to the *Co-1* locus have been previously identified (RAPD: Young and Kelly, 1997a; AFLP: Mendoza et al., 2001). These markers are linked in repulsion phase and have proven to be difficult to reproduce between laboratories. Mendez de Vigo (2001) used the repulsion marker $F10_{530}$ to map the *Co-1* locus to bean linkage group B1. With the advent of the linkage map position of the *Co-1* locus, a series of RAPD (Freyre et al., 1998) and SSR (simple sequence repeat) (Blair et al., 2003) markers located near the locus can now be evaluated for linkage to *Co-1*. The availability of other more reproducible markers linked in coupling phase would greatly facilitate the introgression of the *Co-1* gene into anthracnose resistance gene pyramids using marker-assisted selection (MAS).

Previously, a total of 679 random primers tested in the Cardinal (susceptible)/Kaboon F_2 population, only 15 were polymorphic between DNA bulks and none were found to be linked to the *Co-1* gene (Melotto et al., 2000b). Based on this low level of polymorphism that was found with RAPD analysis in this population, the AFLP technique (Vos et al., 1995) was chosen to develop a molecular marker linked to the *Co-1*[2] gene. It is possible that the *Co-1* gene is flanked by tightly conserved regions with low levels of polymorphisms, which could not easily be detected by the RAPD technique. Additionally, studies have indicated that AFLP analysis is more sensitive to lower levels of polymorphisms than RAPD analysis (Sharma et al., 1996; Mackill et al., 1996). To specifically target the genomic region containing the *Co-1* gene, bulked-segregant analysis (BSA) (Michelmore et al., 1991) was used. The objectives of this study were to identify a molecular marker linked to the *Co-1* locus using RAPD, AFLP, SSR and BSA

analyses and to characterize the anthracnose resistance in the Andean cultivar Jalo EEP558.

MATERIALS AND METHODS

Plant material and *C. lindemuthianum* races. A population segregating for *Co-1*^{2} was used from the cross between Cardinal, a universally susceptible genotype, and Kaboon; this population was designated C/K. Seeds were planted in the greenhouse and tissue was collected from the seedlings at the primary leaf stage for DNA extraction. Approximately ten days after germination, seedlings were spray inoculated with race 73.

The Andean cultivar Jalo EEP558 was inoculated with a series of *C. lindemuthianum* races (Table 1.1). Additionally Jalo EEP558 was crossed with Michigan Dark Red Kidney (MDRK) which carries the *Co-1* gene and Perry Marrow (PM) which carries *Co-1*^{3}. The Jalo EEP558/PM segregating F_2 population was inoculated with race 357, which yields an R/S (resistant/susceptible) reaction in the parents, to determine the number of anthracnose resistance genes in Jalo EEP558. The segregating F_2 population from the Jalo/MDRK cross was used to test for allelism by inoculation with race 73 which is avirulent on both parents.

Inoculum preparation. All races utilitzed in this study were grown from monosporic cultures maintained on filter papers and stored at -20° C. Inoculum was prepared by growing the fungus from a filter paper on either potato dextrose agar (PDA) or Mathur's agar (dextrose (8 g/L), $MgSO_4$. 7 H_2O (2.5 g/L), neopeptone (2.4 g/L), yeast

extract (2 g/L), and agar (16 g/L)) at 22°C in the dark. When the cultures began to sporulate, spores were re-plated in replicate to generate sufficient inoculum. Spores were suspended in a solution of water and 0.01% Tween 20. Spore concentration was estimated using a hemacytometer and the concentration was adjusted to $1.2x10^6$ spores/mL. The prepared inoculum was then sprayed onto the abaxial and adaxial surfaces of the leaves. Inoculated seedlings were incubated at approximately 100% relative humidity for 48 hours. Seedlings were rated for disease 5 days post inoculation on a scale described by Balardin et al. (1997). All races used in this study were first inoculated on the anthracnose differential series to confirm their identities.

AFLP and BSA analyses. Contrasting DNA bulks were constructed by pooling equal volumes of fluoremetrically standardized DNA concentrations from five resistant individuals and five susceptible individuals from the segregating population. The DNA bulks will be referred to as R and S bulks, for resistant and susceptible respectively. The AFLP analysis was conducted as described Hazen et al. (2002). Genomic DNA from parents and bulks was first digested with *Eco*RI and *Mse*I. Adapters which served as subsequent priming sites were ligated to the sticky-ends followed by two PCR amplifications; pre-amplification was performed with one selective base on each primer and selective amplification was performed using primers with three selective nucleotides. Final PCR products were separated on 6% polyacrylamide gels and visualized with silver staining (Promega, Madison, WI).

STS development. The band of interest from each of the parents were cut from a fresh gel using a sterile scalpel. The gel slice was then placed in a microcentrifuge tube with 50 µl of water and incubated overnight at 4°C to diffuse the DNA template. Selective PCR was done with the original selective primer combination using 5 µl of the template DNA to re-amplify the fragment. Various volumes (3, 5, and 10µl) of template DNA were tested to optimize amplification (data not shown). PCR products were then separated on a 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) made with 1XTBE and an ethidium bromide concentration of 0.2 mg/mL. The band was re-isolated from the agarose gel to purify the fragment using a QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified fragment was then cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from transformed bacterial colonies using the Wizard plus SV miniprep kit (Promega, Madison, WI). The plasmid DNA was digested using *Eco*RI and run on a 6% polyacrylamide gel for size selection. Selected colonies containing the inserts of appropriate size were then sequenced at the Genomics Technology Support Facility located at Michigan State University. Sequences were aligned using ClustalW (Thompson et al., 1994) and co-dominant sequence specific primers were designed. The primers were tested on the parents, individuals from the segregating population in addition to other genotypes containing various alleles of the *Co-1* gene and the parents of the mapping population Bat193 and Jalo EEP558. The PCR reaction was as described by Melotto et al. (1996). The thermal cycler profile used was: 3 min at 94 ° C; 30 cycles of 10 s at 94° C, 30 s at 52° C, 2 min at 72° C; 5 min at 72° C.

RAPD analysis. RAPD markers which map near the *Co-1* locus on linkage group B1 were screened on various *Co-1* carrying genotypes (Table 1.2) to determine if any were candidate markers putatively linked to *Co-1*. The RAPD analysis was similar that described by Haley et al. (1993) using Taq DNA polymerase (Invitrogen, Carlsbad, CA) and Operon primers (Operon Technologies, Inc., Alameda, CA). The thermal cycler profile used was 3 cycles of 1 min at 94° C, 1 min at 35° C, 2 min at 72° C; 34 cycles of 10 s at 94° C, 20 s at 40° C, 2 min at 72° C; 5 min at 72° C. PCR products were run on 1.4% agarose gels using 1X TAE and 0.2 mg/mL of ethidium bromide.

SSR analysis. SSR markers located on linkage group B1 (Table 1.3) were tested on parents and R and S bulks. The PCR reaction contained 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 μM of each primer, 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 50 ng DNA. The thermal cycler profile used was 5 min at 94° C; 30 cycles of 1 min at 94° C, 1 min at 47° C, 1 min at 72° C; 5 min 72° C. PCR products were separated on 6% polyacrylamide gels as described in AFLP analysis above.

Molecular Mapping. Candidate AFLP markers and the STS marker, $SE_{ACT}M_{CCA}$, were tested for linkage to resistance on the C/K segregating population using MAPMAKER (Lander et al. 1987). Linkage distance in cM was calculated using Kosambi's mapping function with a LOD threshold value of 2.0. $SE_{ACT}M_{CCA}$ was also mapped to the bean integrated linkage map using 65 recombinant inbred lines (RILs). These RILs are a subset of those used originally to generate the integrated map from the F_2 population BAT93/Jalo EEP558 (Freyre, 1998).

RESULTS AND DISCUSSION

RAPD, SSR and AFLP analyses. One method employed to identify markers linked to the *Co-1* locus was to test markers located near the previously mapped $F10_{530}$ RAPD marker (Young and Kelly, 1997) linked to the *Co-1* locus. Nine RAPD and seven SSR markers were tested for putative linkage to the *Co-1* locus using parents, R and S bulks in addition to several genotypes carrying varying alleles at the *Co-1* locus. None of the RAPD and SSR markers tested, however, appeared to be associated with the presence of the *Co-1* locus (Data not shown). A total of 232 AFLP primer pair combinations were tested for linkage with the *Co-1* locus. 22 primer combinations (10.5% of all primers tested) were polymorphic between parents and R and S bulks, however, only one, $E_{ACT}M_{CCA}$, showed putative linkage when tested on the individual members of the R and S bulks (Figure 1.1A). Two bands of interest are produced among the PCR products which result from the $E_{ACT}M_{CCA}$ primer combination. The larger band is 108 bp in length and is present in Kaboon and the R bulk, suggesting a putative linkage to the resistance conferred by the $Co\text{-}1^2$ gene. The smaller band is present in the susceptible parent, Cardinal, and the S bulk indicating that it may be linked to the susceptible allele at the $Co\text{-}1^2$ locus. The linkage analysis revealed that this marker, $E_{ACT}M_{CCA}$-108/107 was linked at a distance of 9.9 cM from the *Co-1* locus and segregated in a 1:2:1 (p=0.478) ratio indicating that it is a co-dominant marker. The marker was tested on cultivars BAT93 and Jalo EEP558, the parents of a mapping population and genotypes with different *Co-1* alleles (Figure 1.1B). The band associated with the *Co-1* locus (108 bp),

however, was not amplified in these genotypes possibly due to mutations in the restriction sites of the enzymes and thus, $E_{ACT}M_{CCA}$-108/107 appears to be specific to the *Co-1*^{2} allele of the *Co-1* locus.

STS marker. AFLP analysis is relatively complicated and costly, requiring extensive template preparation. Consequently, AFLP markers are not well suited for marker-assisted selection (MAS) of large populations in breeding programs. To facilitate the use of MAS to introgress the *Co-1*^{2} gene into susceptible cultivars, the AFLP marker was converted to a sequence-tagged site (STS) marker. Figure 2 shows the sequence alignment of the resistant and susceptible alleles of the marker. The alignment of the sequences revealed that the two bands differ only by two changes; a one bp deletion which results in a fragment length difference and a single nucleotide polymorphism. Due to the small size of the original AFLP fragments, only 107 and 108 bp, the derived STS marker, $SE_{ACT}M_{CCA}$, was also very small, 79 and 80 bp. The co-dominant nature and the small fragment length difference between alleles of the $SE_{ACT}M_{CCA}$ marker requires the use of polyacrylamide gels as the method to resolve the PCR products. This requirement and the distance of the marker from the locus, 9.9 cM, makes it less suitable for use in MAS.

The derived STS marker was also tested on genotypes which carry different alleles at the *Co-1* locus (Figure 1.2). The allele of $SE_{ACT}M_{CCA}$ which is associated with resistance (108 bp), is also present in cultivars MDRK (*Co-1*) and Perry Marrow (*Co-1*^{3}). Thus, in contrast to the AFLP marker, $E_{ACT}M_{CCA}$-108/107, which appears to be allele specific, the derived STS marker is not allele specific. This is most likely because the

STS primers do not include the entire restriction enzyme site, therefore, mutations in these sites do not affect the amplification of $SE_{ACT}M_{CCA}$. Although $E_{ACT}M_{CCA}$-108/107 and $SE_{ACT}M_{CCA}$ are not suitable for MAS, these two markers can be used to initiate the characterization of Andean sources or anthracnose resistance. $SE_{ACT}M_{CCA}$ can be used to detect the presence of resistant alleles at the *Co-1* locus; $E_{ACT}M_{CCA}$-108/107 can be used to identify the *Co-1*^{2} allele within Andean germplasm. No association was detected between the presence of either marker and the *Co-1* locus among Middle American genotypes tested (Data not shown) and thus the use of these markers is believed to be limited to Andean germplasm.

To confirm the genomic location of the *Co-1* locus, $SE_{ACT}M_{CCA}$ was mapped using a RIL population derived from the cross between genotypes Bat93 and Jalo EEP558. No amplification product was observed in BAT93; this is not surprising considering BAT93 is a Middle American cultivar. Jalo EEP558 presented the 80 bp fragment, therefore the marker was scored in a dominant fashion rather than co-dominant. The linkage analysis confirmed the placement of the *Co-1* locus on linkage group B1 (Figure 1.3). Based on linkage of *Co-1* with the RFLP clones used to construct and integrate the physical map with the genetic linkage map of common bean (Pedrosa et al., 2003), the *Co-1* locus is located on the long arm of chromosome 2.

Polymorphism at *Co-1* genomic region. The region surrounding the *Co-1* locus appears to have limited variability at the DNA level. Support for this observation comes from the small number of polymorphisms detected between the R and S bulks, only 10.5 % of 232 primers tested. One theory is that this is a result of the genomic location of the

Co-1 locus. The *Co-1* locus is located on the distal end of linkage group B1, adjacent to the largest block of genes which control the domestication syndrome in the bean genome (Koinange et al., 1996). Blocks of domestication genes, much like co-adaptive gene complexes, are highly conserved. The population used in this study was between two domesticated bean lines, thus little variability between the parents is expected at the block of domestication genes. Since the *Co-1* locus maps adjacent to this block of highly conserved genes, it is not surprising that reduced polymorphism was detected surrounding this locus.

Characterization of anthracnose resistance in Jalo EEP558. To characterize the anthracnose resistance in the Andean cultivar Jalo EEP558, Jalo EEP558 was first screened with markers linked to other known anthracnose resistance genes (Table 1.4). Only $SE_{ACT}M_{CCA}$ 80 was present in Jalo EEP558, indicating that Jalo EEP558 carries the *Co-1* gene. The disease reaction of Jalo EEP558, when inoculated with 10 diverse races of *C. lindemuthianum*, is identical to that of MDRK (Table 1.1). The comparison of the disease reaction of Jalo EEP558 with that of other *Co-1* containing cultivars supports the hypothesis that Jalo EEP558 carries the *Co-1* locus and suggests that it is the same allele present in the differential cultivar MDRK. To determine the number of anthracnose resistance genes segregating from Jalo EEP558, 94 plants from the Jalo EEP558/PM F_2 population were inoculated with race 357, which gives an R/S (resistant/susceptible) reaction in the parents (Table 1.5). The segregation ratio of resistant:susceptible individuals of the Jalo EEP558/PM population fits both a 13:3 ratio (p-value = 0.667) and a 3:1 ratio (p-value = 0.074). The population size used in this experiment prevents us

from distinguishing between a 13:3 or a 3:1 ratio. A segregation ratio of 13:3 suggests that Jalo EEP558 carries one dominant and one recessive gene with epistasis. A ratio of 3:1 suggests that only one dominant gene conditions resistance to race 357. Therefore, we are unable to determine if Jalo EEP558 carries more than one gene which conditions resistance to anthracnose. Interestingly, Geffroy et al. (1999), identified two putative Andean anthracnose resistance genes (*Co-y* and *Co-z*) from Jalo EEP558. These loci map to a gene cluster which contains the Middle American resistance gene *Co-9* on linkage group B4 of the integrated map. This gene cluster, comprising both Andean and Middle American anthracnose resistance genes, suggests that this locus existed prior to the separation of the two major gene pools of *P. vulgaris*. The authors did not characterize their fungal strains by the universal anthracnose differential series and thus the results of this study cannot be compared to other studies.

To test the anthracnose resistance in Jalo EEP558 for allelism with the *Co-1* locus, 200 individuals from the Jalo EEP558/MDRK F_2 population were inoculated with *C. lindemuthianum* race 73, which elicits an R/R (resistant/resistant) reaction in the parents (Table 1.5). No segregation in the progeny was observed indicating that Jalo EEP558 carries the *Co-1* gene or a cluster gene family member.

CONCLUSIONS

The *Co-1* locus offers breeders choices among alleles for anthracnose resistance, however, other Andean resistance genes must be characterized to provide diverse tools necessary for developing more durable anthracnose resistance. Although many Andean

sources of anthracnose resistance have been characterized, no new resistance loci are being identified. This begs the question: Why are there so many independent anthracnose resistance genes in the Middle American gene pool when compared to the Andean gene pool? The answer to this interesting question may lie in an understanding of the domestication of *P. vulgaris*. Common bean is a noncentric crop, having two or more domestication events in distinct wild populations leading to the formation of two geographically separated gene pools of domestication. The cultivated Andean gene pool, however, has a very narrow genetic base when compared to Middle American. Beebe et al. (2001) suggest that the three Andean bean races share a common origin based on the multiple correspondence analysis of AFLP genotyping of landrace, wild and cultivated beans of Andean and Middle American origin. This narrow genetic base suggests that the domestication of Andean beans occurred within a narrow wild population (Beebe et al., 2001). This has direct implications on the diversity of independent anthracnose resistance genes available within the cultivated Andean gene pool. The identification of multiple resistance genes in the Andean cultivar Jalo EEP558, suggests that uncharacterized resistance to anthracnose in the Andean gene pool remains to be discovered.

The apparent deficiency of polymorphism at the molecular level in the genomic region of the *Co-1* locus, which may be a result of close linkage to a block of genes contributing to the domestication syndrome, has made the identification of markers linked to this locus very difficult. To circumvent this problem a population derived from the cross of a cultivated by a wild bean should be used. Molecular markers linked to the

Co-1 locus which can differentiate between all alleles will be useful to facilitate the introgression of anthracnose resistance conditioned by this complex locus.

Table 1.1 Disease reaction[a] of Jalo EEP558 and three differential cultivars to 10 races of *C. lindemuthianum*.

Race	Jalo EEP558	MDRK[b]	PM[c]	Kaboon[d]
2	S	S	R	R
7	S	S	S	R
38	S	S	S	S
47	S	S	S	S
73	R	R	R	R
80	R	R	R	R
88	R	R	R	R
128	R	R	R	R
357	R	R	S	S
1545	R	R	R	R

[a] Disease reaction: R = resistant, S = susceptible

[b] *Co-1*

[c] *Co-1*^{3}

[d] *Co-1*^{2}

Table 1.2 RAPD markers located near the mapped location of the *Co-1* locus tested on bean cultivars with different anthracnose resistance genes.

RAPD Markers	Bean Cultivars	Anthracnose Resistance Gene
$J4_{400}$	Cardinal	No known anthracnose resistance genes
$D20_{1000}$	La Victoria	No known anthracnose resistance genes
$H3_{1100}$	Kaboon	*Co-1*^{2}
$D8_{800}$	MDRK	*Co-1*
$A13_{625}$	Perry Marrow	*Co-1*^{3}
$F6_{400}$	Cornell 49-242	*Co-2*
$A4_{700}$	BAT93	*Co-9*
$D6_{800}$	Jalo EEP558	Characterized in current study
$F6_{800}$		

Table 1.3 SSR markers evaluated for putative linkage with the *Co-1* locus using cultivars Cardinal and Kaboon and R and S bulks.

SSR markers (expected size bp)
BM146 (281)[b]
BM 157 (113)[b]
BMd-10 (139)[a]
Pv-ag003 (201)[c]
BM 53 (287)[b]
BM 200 (221)[b]
BMd-45 (129)[a]

[a] Primer sequences are available in Blair et al. (2003).

[b] Primer sequences are available in Gaitán-Solís et al. (2002).

[c] Primer sequences are available in Yu et al. (2000).

Table 1.4 Molecular markers linked to known anthracnose resistance genes tested on Jalo EEP558.

Molecular Marker	Linked Anthracnose Locus	Reference
$SAB3_{500}$	*Co-5*	Chapter Three
$SH18_{1200}$	*Co-4*^{2}	(Awale and Kelly, 2001)
$SAS13_{950}$	*Co-4*	(Young et al., 1998)
$SE_{ACT}M_{CCA}$	*Co-1*	Present Chapter

Table 1.5 F2 populations inoculated with *C. lindemuthianum*.

Population	Race Inoculated	Disease Reaction	Observed Ratio[a]	Expected Ratio[a]	p-value
Jalo EEP558/PM	357	R/S	78:16	13:3	0.667
				3:1	0.074
Jalo EEP558/MDRK	73	R/R	200:0	-	-

[a] Ratio of resistant to susceptible individuals

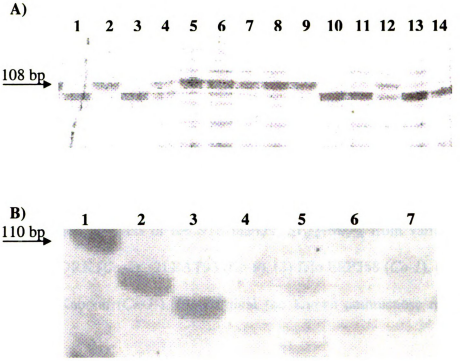

Figure 1.1 Amplification products of the AFLP marker $E_{ACT}M_{CCA}$-108/107 from various bean genotypes using **A)** (1) Cardinal, (2) Kaboon, (3) susceptible bulk, (4) resistant bulk, (5-9) individuals of the resistant bulk, (10-14) individuals of the susceptible bulk, **B)** (1) 110 bp fragment of the 10 bp ladder, (2) Kaboon, (3) Cardinal, (4) Jalo EEP558, (5) BAT93, (6) MDRK, (7) Perry Marrow.

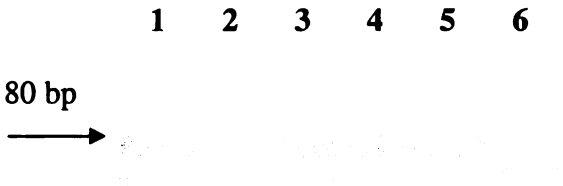

Figure 1.2 Amplification products of the STS marker, $SE_{ACT}M_{CCA}$ from various bean genotypes using (1) MDRK (*Co-1*), (2) BAT93 (*Co-9*), (3) Jalo EEP558 (*Co-1*), (4) Perry Marrow (*Co-1*^{3}), (5) Kaboon (*Co-1*^{2}), (6) Cardinal (no known anthracnose resistance genes).

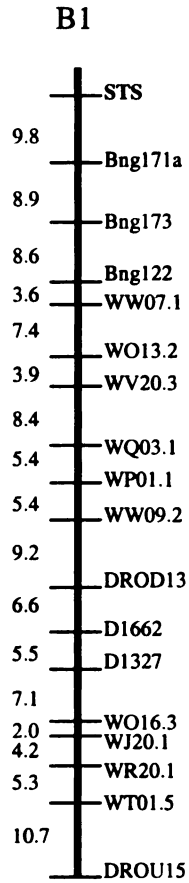

Figure 1.3 The STS marker $SE_{ACT}M_{CCA}$ was mapped to bean linkage group B1 using the BAT93/Jalo EEP558 RIL population.

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CHAPTER TWO

MOLECULAR AND GENETIC CHARACTERIZATION OF THE *CO-7* LOCUS CONDITIONING RESISTANCE TO ANTHRACNOSE IN COMMON BEAN AND BREEDING LINE MSU7

ABSTRACT

The most resistant cultivar in the anthracnose differential series is the landrace G 2333. G 2333 is known to carry a three gene pyramid for anthracnose resistance: *Co-4*^{2}, *Co-5* and *Co-7*. Both *Co-5* and *Co-4*^{2} genes have been well characterized through the use of lines derived from crosses with G 2333 that carry these individual genes. The objectives of this study were to characterize the spectrum of resistance conditioned by the *Co-7* gene and to develop a molecular marker linked to this resistance gene. In order to characterize resistance conferred by this locus, the *Co-7* gene had to be separated from the other anthracnose resistance genes. To this purpose, the F_2 population from the cross Black Magic (susceptible)/Sel 111 (resistant and carries *Co-5* and *Co-7*) was inoculated with *C. lindemuthianum* race 7. The resistant individuals would carry *Co-7* (homozygous or heterozygous) and/or *Co-5* (homozygous or heterozygous). To select against the *Co-5* gene, in the absence of a race to discriminate between *Co-5* and *Co-7*, the SCAR marker, SAB3 was developed from the RAPD marker $AB3_{450}$ which is linked to the *Co-5* locus. F3 progeny tests conducted with race 7 identified those individuals which were homozygous at the *Co-7* locus and these were designated MSU7-1 through -6. I confirmed that MSU7 carried only one gene conditioning resistance to race 7 by crossing MSU7 with MDRK and inoculating the F_2 (3:1 segregation ratio with p-value = 0.82).

Also, lines MSU7-3, MSU7-4 and MSU7-6 were all resistant to race 7 and susceptible to races 448 and 73, suggesting that they carry only the *Co-7* gene. Inoculation with additional *C. lindemuthianum* races revealed that line MSU7-3 carries partial resistance to races 31 and 47. Based on these results it is clear that *Co-7* does not contribute significantly to the resistance spectrum of G 2333, although epistatic interactions were not investigated in this study. The main value of the *Co-7* gene for breeding for anthracnose resistance will be in the large-seeded Andean varieties of dry bean which are generally very susceptible to race 7 of *C. lindemuthianum*. An amplified fragment length polymorphism (AFLP) marker was developed, which mapped 4.9 cM from the *Co-7* locus, to facilitate the introgression of the *Co-7* gene into susceptible bean cultivars.

INTRODUCTION

Anthracnose caused by the fungal pathogen *Colletotrichum lindemuthianum*, is a serious seed-borne disease of dry beans. Worldwide, yield losses due to anthracnose are particularly severe in subsistence agricultural systems where beans are a valuable source of plant protein, and income to the producer. Genetic resistance is the most cost effective means to control the disease. Ten major anthracnose resistance genes (*Co-1* – *Co-10*) have been characterized in common bean and are available for use in breeding programs (Kelly and Vallejo, 2004). Achieving durable anthracnose resistance poses a challenge to bean breeders. Due to the high degree of pathogen variability and the continual emergence of new races, single gene deployment is not an effective strategy to control bean anthracnose. The pyramiding of resistance genes which have complementary spectra of resistance has been suggested as a strategy to circumvent the problem of pathogen variability (Young and Kelly, 1996a; Young and Kelly, 1996b).

The most resistant Middle American cultivar in the anthracnose differential series is G 2333. G 2333 is the landrace cultivar, Colorado de Teopisca from Chiapas, Mexico that possesses a naturally occurring gene pyramid for anthracnose resistance. The first report on the nature of resistance in G 2333 suggested that two independent dominant genes were present. A 15:1 (resistant:susceptible) ratio was observed after inoculation of an F_2 population of G 2333/Pijao with race 521 (Pastor-Corrales et al., 1994). Young and Kelly (1996a) identified the first gene in G 2333 as *Co-5* using breeding line SEL 1360 derived from G 2333 (Young and Kelly, 1998). They inferred that since race 521, used by Pastor-Corrales et al. (1994), overcomes the *Co-5* gene G 2333 must possess three

independent dominant resistance genes, one of which is *Co-5*. The second gene in G 2333 was discovered to be allelic to the *Co-4* gene in differential cultivar TO and designated *Co-4*^{2} (Young et al., 1998). The breeding line SEL 1308, derived from G 2333, carries only the *Co-4*^{2} gene from the original gene pyramid. The third anthracnose resistance gene from G 2333 was designated *Co-7* (Young et al., 1998), however, no further characterization was performed and little is known about the spectrum of resistance which it confers.

The availability of lines SEL 1308 and SEL 1360 facilitated the identification of the *Co-4*^{2} and *Co-5* genes respectively in G 2333. These lines permited the characterization of the anthracnose resistance conditioned by the genes and provided breeders with information on which to base their gene combinations and deployments strategies. Additionally, these lines were used to identify markers linked to their respective anthracnose resistance genes which serve as tools to expedite the introgression process (Young and Kelly, 1998). To begin to isolate the *Co-7* gene from the other anthracnose resistance genes in the pyramid, Young et al. (1998) used a combination of marker-assisted selection (MAS) and inoculation with races of *C. lindemuthianum* to derive SEL 111, which possessed two anthracnose resistance genes: *Co-5* and *Co-7*. The goals of the current study were to (1) derive a bean breeding line which only carries the *Co-7* gene for anthracnose resistance using a combination of inoculation with *C. lindemuthianum* races and MAS, (2) characterize the resistance spectrum conditioned by the *Co-7* gene and (3) identify a molecular marker linked to the *Co-7* gene using amplified fragment length polymorphism (AFLP) and bulked-segregant (BSA) analyses.

MATERIALS AND METHODS

Plant Material and *C. lindemuthianum* races. Populations developed in this study are shown in Table 2.1. To confirm that the original breeding line SEL 111 carries only two major genes for anthracnose resistance, a segregating F_2 population was derived from the cross between the cultivar Black Magic (BM) and SEL 111. The black bean BM is generally considered universally susceptible to anthracnose. This F_2 population of 72 individuals was inoculated with race 7 which produced an S/R (susceptible/resistant) reaction in the parents (BM and SEL 111 respectively). A molecular marker linked to the *Co-5* locus was used to select against the presence of the *Co-5* gene among the progeny segregating for the *Co-5* and *Co-7* loci within the BM/SEL 111 population. A F_3 progeny test was conducted to determine which of the F_2 individuals, which showed the absence of the *Co-5* locus based on MAS, were homozygous for *Co-7* by inoculation of F_3 families of 15 individuals each, with race 7. The derived lines were named MSU7-1 through 6 and were advanced through five generations of selfing. Line MSU7-5 was not used in this study because of insufficient seed. These $F_{2:5}$ MSU7 lines were inoculated with a series of *C. lindemuthianum* races (7, 31, 47, 73, 357, 448 and 1545) to determine the resistance spectrum conferred by the *Co-7* gene. Additionally, specific $F_{2:5}$ MSU7 lines were crossed with the cultivar Michigan Dark Red Kidney (MDRK) and the resulting F_2 population was inoculated with race 7 which yields an S/R reaction in the parents (MDRK and MSU7 respectively) to determine the number of genes segregating for anthracnose resistance.

Inoculum preparation and inoculation procedures. All races utilitzed in this study were grown from monosporic cultures maintained on filter papers and stored at -20° C. *C. lindemuthianum* races were grown from a filter paper on either potato dextrose agar (PDA) or Mathur's agar (dextrose (8 g/L), $MgSO_4$. 7 H_2O (2.5 g/L), neopeptone (2.4 g/L), yeast extract (2 g/L), and agar (16 g/L)) at 22°C in the dark. After the onset of sporulation, spores were re-plated in replicate to generate sufficient inoculum. Spores were scraped from the media into a solution of water and 0.01% Tween 20. An estimated spore concentration was determined using a hemacytometer and the concentration was adjusted to 1.2 x 10^6 spores/mL. The prepared inoculum was then sprayed onto the abaxial and adaxial surfaces of the leaves. Inoculated seedlings were incubated for 48 hours at approximately 100% relative humidity. Seedlings were rated for disease 5 days post inoculation on a scale described by Balardin et al. (1997). All races used in this study were first inoculated on the anthracnose differential series to confirm their identities.

MAS analysis and derivation of a SCAR marker. To dissect the *Co-5*, *Co-7* gene pyramid in SEL 111, the BM/SEL 111 segregating population was inoculated with race 7, and a molecular marker linked to the *Co-5* locus was used in a MAS strategy. The RAPD marker, $AB3_{450}$, is linked in coupling-phase to *Co-5* (5.9 ± 1.9 cM) (Young and Kelly, 1997). This marker was converted into a sequence characterized amplified region (SCAR) marker, using the protocol described by Melotto et al. (1996), to facilitate its use in a MAS approach. Primer sequences for $SAB3_{450}$ are shown in Table 2.2. The thermal

cycler profile used to amplify the SAB3 marker consisted of 34 cycles of 10 s at 94°C, 40 s at 67°C, and 2 min at 72°C; one cycle of 5 min at 72°C.

AFLP and BSA analyses. DNA bulks (Michelmore et al., 1991) were constructed by pooling equal volumes of fluoremetrically standardized DNA concentrations from six resistant individuals and six susceptible individuals from the segregating MDRK/MSU7 population. The DNA bulks will be referred to as R and S bulks, for resistant and susceptible respectively. The AFLP analysis was conducted as described Hazen et al. (2002). Briefly, genomic DNA from parents and bulks was digested with *Eco*RI and *Mse*I. Adapters which serve as subsequent priming sites were ligated to the sticky-ends followed by two PCR amplifications, pre-amlification was done with one selective base on each primer and selective amplification was done using primers with three selective nucleotides. Final PCR products were separated on 6% polyacrylamide gels and visualized with silver staining (Promega, Madison, WI).

Linkage analysis. Candidate AFLP markers were tested for linkage to resistance on the MDRK/MSU7 segregating population using MAPMAKER 3.0 (Lander et al. 1987). Linkage distance in cM was calculated using Kosambi's mapping function with a LOD threshold value of 2.0.

RESULTS AND DISCUSSION

Derivation and genetic characterization of MSU7 lines. To confirm that SEL 111 carried two major genes for resistance to anthracnose, SEL 111 was crossed with BM

and the resulting F_2 population was inoculated with race 7, which yields and S/R (susceptible/resistant) reaction in the parents (BM and SEL 111 respectively). The segregation of resistant to susceptible individuals fit a 15:1 ratio ($p = 0.57$) indicating that two dominant anthracnose resistance genes were segregating from SEL 111 for resistance to race 7 (Table 2.2).

To isolate *Co-7* from *Co-5*, the SCAR marker $SAB3_{400}$ was developed to select within the segregating population against the *Co-5* locus. The derived SCAR marker amplified a 400 bp fragment that appears as a single polymorphic band between the parents (data not shown). Young and Kelly (1997) reported a band of 450 bp linked to the *Co-5* locus, however, I consistently amplified a band 400 bp in size with the RAPD marker, $OAB3_{450}$ and the derived SCAR marker, $SAB3_{400}$.

Using $SAB3_{400}$ to indirectly select against the *Co-5* locus, all of the individuals from the BM/SEL 111 F_2 population which were resistant to race 7 were screened. Those individuals which did not carry $SAB3_{400}$ were used for further study. F_3 families of these individuals were subjected to a progeny test in which 15 $F_{2:3}$ seedlings were inoculated with race 7 to determine which F_2 individuals were homozygous for *Co-7*. Those F_2 individuals, whose F_3 family did not segregate for resistance when inoculated with race7, were self-pollinated for two additional generations and designated breeding lines MSU7-1 through 6.

To confirm the existence of a single dominant gene conferring resistance to race 7, lines MSU7-3 and MSU7-4 were used for crossing with MDRK. All F_2 seeds from these crosses were planted as a single population (referred to collectively as MDRK/MSU7), however, pedigrees were recorded to facilitate the removal of progeny

which resulted from self-pollination. The F_2 population from the MDRK/MSU7 crosses was inoculated with race 7. The segregation of resistant to susceptible individuals best fits a 3:1 ratio ($p = 0.82$) indicating that only one major dominant gene is conferring resistance to race 7 in MSU7-3 and MSU7-4 (Table 2.2).

Resistance conditioned by the *Co-7* gene. Initial characterization of the MSU7 lines was done by inoculation with *C. lindemuthianum* races: 7, 73 and 448. The disease reaction profile of the MSU7 lines was compared with SEL 111 and SEL 1360 (Table 2.3). Inoculation of the MSU7 lines with these three races revealed that MSU 7-1 had the same disease reaction as SEL 111 and SEL 1360. Lines MSU 7-3, 7-4, and 7-6, however, were susceptible to races 73 and 448. These results suggest that MSU 7-1 is more resistant than lines MSU7-3, MSU7-4, and MSU7-6. The fact that the resistance spectrum of MSU 7-1 is identical to that of SEL 111 (*Co-5* and *Co-7*) and SEL 1360 (*Co-5*) suggests that MSU 7-1 carries *Co-5* or both *Co-5* and *Co-7* and is a result of a recombination event between $SAB3_{400}$ and the *Co-5* locus. MSU7-2 showed segregation within the line when inoculated indicating possible seed contamination (data not shown), therefore, this line was removed from this study. Lines MSU7-3, MSU7-4, and MSU7-6 were resistant to race 7 and susceptible to races 73 and 448, indicating that they carry only *Co-7*. Support for this finding comes from a study by Alzate-Marin et al. (1998) who reported a 15:1 segregation ratio of resistant to susceptible individuals in a F_2 population from the cross G 2333/Ruda inoculated with race 73. Since Ruda does not carry major genes for anthracnose resistance, and G 2333 is known to carry three, one of the genes must have been overcome by race 73. Neither SEL 1308 ($Co\text{-}4^2$) or SEL 1360

(*Co-5*) confer susceptibility to race 73 (Kelly and Vallejo, 2004), therefore, *Co-7* must then condition susceptibility to race 73.

Further characterization of the anthracnose resistance conditioned by the *Co-7* gene was conducted by inoculating MSU7-3, MSU7-4 and MSU7-6 with additional *C. lindemuthianum* races: 31, 47, 357 and 1545 (Table 2.3). All MSU7 lines inoculated with race 1545 were susceptible. Race 357 overcame resistance in all three MSU7 lines tested but not the resistance in SEL 111 and SEL 1360, indicating that the resistance in SEL 111 to race 357 is conferred by the *Co-5* gene and not *Co-7*. Inoculation with races 31 and 47 showed that MSU7-3 possessed partial resistance when compared to MSU7-4 and MSU7-6 which were susceptible. The author defines partial resistance as the appearance of very small constricted lesions that do not lead to plant death. This observed difference between the disease reactions of the MSU7 lines to the same race of *C. lindemuthianum* can be explained by differences in the genetic background among the lines

Molecular marker analysis. A total of 147 AFLP primer pair combinations were tested on the parents and R and S bulks from the MDRK/MSU7 F_2 population. Five candidate markers linked to *Co-7* were identified. Candidate markers were defined by the same polymorphism being present in the parents and contrasting DNA bulks. Only one primer pair, $E_{ACC}M_{CGT}$-290, however, was linked to the *Co-7* locus (Figure 2.1). Linkage analysis revealed that the AFLP marker, $E_{ACC}M_{CGT}$-240, was linked in repulsion phase to the *Co-7* locus at 4.9 cM. The *Co-5* and *Co-7* loci could not be mapped to the bean integrated map (Freyre et al., 1998) due to lack of polymorphism between the parents of the mapping population: BAT93, Jalo EEP558 (data not shown).

CONCLUSIONS

The *Co-7* gene conditions resistance to race 7, but susceptibility to all other races tested. This indicates that the *Co-7* gene does not contribute significantly to the resistance spectrum of G 2333, although epistatic interactions have not been tested. Race 7 is a widespread race of *C. lindemuthianum* and a particular problem in large-seeded Andean varieties of dry bean. I suggest that *Co-7* be used in combination with other resistance genes with complimentary spectra for anthracnose resistance breeding of the large-seeded Andean bean varieties such as kidney and cranberry beans.

In this study, the dissection of a gene pyramid was accomplished through a MAS and inoculation strategy. A caveat of working with molecular markers for the dissection of gene pyramids is the potential for selecting recombinant individuals, that is, individuals in which the molecular marker and the resistance allele have been uncoupled as a result of a recombination event. In the present study, I identified line MSU7-1 which has the same disease reaction as SEL 111 and SEL 1360 indicating that it still carries the *Co-5* gene and possibly the *Co-7* gene, but which does not carry the marker, $SAB3_{400}$ linked to the *Co-5* locus. Unfortunately, I have not yet tested MSU7-1 for the presence of the marker, $E_{ACC}M_{CGT}$-240, to test for the *Co-7* gene. MSU7-2 appears to be more resistant than MSU7-3 and MSU7-6, conferring partial resistance to races 31 and 47, both races of Andean origin. I speculate that the partial resistance is due to background genetic effects between the different MSU7 lines. I propose that lines MSU7-3 and MSU7-6 are homozygous for *Co-7* based on molecular marker data and inoculation with different races of *C. lindemuthianum*. The availability of the MSU7 lines permitted the

characterization of the anthracnose resistance conditioned by the *Co-7* gene and the identification of a marker linked to this locus. This marker and the MSU7 lines provide breeders tools to expedite the introgression of the *Co-7* gene into a complementary gene pyramid. Additionally, since the *Co-7* gene has never been fully tested for independence of all known anthracnose resistance loci, the availability of the MSU7 lines should facilitate the full characterization of the *Co-7* locus.

Table 2.1 Primer sequences for the SCAR marker $SAB3_{400}$ linked to the *Co-5* locus.

Forward primer sequence[a]	5'-TGGCGCACACATAAGTTCTCACGG-3'
Reverse primer sequence[a]	5'-TGGCGCACACCATCAAAAAAGGTT-3'

[a] The underlined sequences are the original RAPD primer.

Table 2.2 F_2 Populations inoculated with *C. lindemuthianum* race 7.

Population	Parental Reaction	Population Size	Observed ratio	Expected ratio	P-value
BM/SEL 111	S/R	155	147:8	15:1	0.57
MDRK/MSU7[a]	S/R	55[b]	42:13	3:1	0.82

[a] Lines MSU7-3, MSU7-4 were used to develop this population

[b] Population size is small because crosses with MSU7-2 had to be removed from population due to evidence of seed contamination.

Table 2.3 Disease reaction of MSU7 lines derived from G 2333 and SEL 111 and SEL 1360 inoculated with various races of *C. lindemuthianum*.

		C. lindemuthianum races						
Breeding line	R-gene(s)	7	73	448	31	47	357	1545
MSU7-1	*Co-5*, *Co-7*	R	R	R	NT[a]	NT[a]	NT[a]	NT[a]
MSU7-2	NA[b]	R	R	S	NT[a]	NT[a]	NT[a]	NT[a]
MSU7-3	*Co-7*	R	S	S	R[c]	R[c]	S	S
MSU7-4	*Co-7*	R	S	S	S	S	S	S
MSU7-6	*Co-7*	R	S	S	S	S	S	S
SEL 111	*Co-5*, *Co-7*	R	R	R	R	R	R	S
SEL 1360	*Co*-5	R	R	R	R	R	R	S

[a] NT: not tested

[b] NA: information not available

[c] R: partial resistance

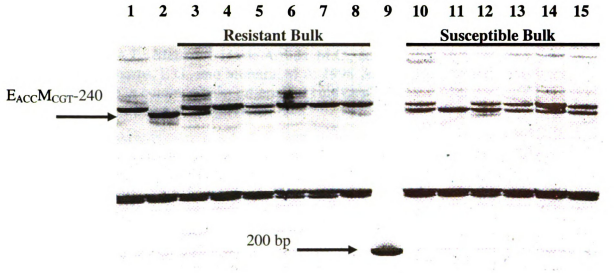

Figure 2.1 Partial gel of amplification products produced by AFLP primer pair $E_{ACC}M_{CGT}$ tested on parents and individuals of resistant and susceptible bulks. Lanes (1) MSU7-3, (2) MDRK, (3-8) individuals from resistant bulk, (9) 100 bp ladder, (10-15) individuals from susceptible bulk. Upper arrow indicates the lower band as the marker linked to *Co-7*. Lower arrow indicates the 200 bp fragment of the 100 bp ladder.

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CHAPTER THREE

ANALYSIS OF CONSTITUTIVELY EXPRESSED AND INDUCED GENES IN THE RESPONSE OF *PHASEOLUS VULGARIS* TO *COLLETOTRICHUM LINDEMUTHIANUM*

ABSTRACT

The induction of the defense response in plants triggers a rapid multicomponent series of events. Initial response of the plant can occur within minutes and involve rapid gene activation. The response of common bean, *Phaseolus vulgaris*, to an incompatible race of *Colletotrichum lindemuthianum* was used in this study. *C. lindemuthianum* is the fungal pathogen which causes bean anthracnose, a serious seed-borne disease of dry bean worldwide. The objectives of this study were to use cDNA-AFLP to determine if any constitutively expressed differences exist between plants which carry the *Co-4*^{2} gene that may contribute to the resistance response, and (2) identify genes which are differentially regulated during the early stages of the incompatible interaction between *C. lindemuthianum* and *P. vulgaris*. Several constitutively expressed genes involved in primary metabolism were discovered which have a possible role in the ability of the bean plant to mount *Co-4*^{2} mediated resistance. Additionally, several genes were identified which are differentially regulated between inoculated and mock-inoculated treated plants in the incompatible interaction. Changes in gene expression were detected as early as 1 hour post inoculation. Among the genes identified, was a putative serine/threonine receptor kinase, a putative lysine decarboxylase-like enzyme and a NIM1-like protein. The products of these genes have previously been correlated with the defense response in a number of other taxa. I speculate that the combination of constitutive expression

differences in primary metabolism processes and genes involved in active defense contribute to anthracnose resistance mediated by the *Co-4*^{2} gene in common bean.

INTRODUCTION

Resistance to pathogens in plants is accompanied by the rapid deployment of a multicomponent defense response. Individual components of this response include the hypersensitive response (HR), chemicals with antimicrobial properties such as phytoalexins and hydrolytic enzymes and structural changes such as the lignification and strengthening of cell walls (Dixon and Harrison, 1994). Initial response of pathogen-challenged or elicitor-treated plant cells can occur within minutes and involve rapid gene activation. The intracellular signal transduction events during the initial stages of the resistance response include rapid and transient changes in ion fluxes across the plasma membrane, production of oxidative bursts and changes in the phosphorylation status of various proteins (Kombrink and Schmelzer, 2001). Thus, extensive reprogramming of both primary and secondary metabolism occurs at the level of gene expression (Schenk et al., 2000) and many of the induced proteins have a direct or indirect inhibitory effect on the pathogen (Hammond-Kosack and Jones, 1996).

The incompatible interaction between *C. lindemuthianum*, the fungal pathogen which causes anthracnose on dry bean (*Phaseolus vulgaris*), has been previously studied at the level of cytology and gene expression of key enzymes involved in the defense response (Ryder et al., 1984; Edwards et al., 1985; Cramer et al., 1984; Bell et al., 1986; Hedrick et al., 1988; Mahé et al., 1992; Fraire-Velázquez and Lozoya-Gloria, 2003). These studies have primarily focused on the induction of the L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes either in cell suspension or whole plants in response to fungal inoculation, fungal-derived elicitors or chemical elicitors. PAL and CHS are key enzymes in the production of phytoalexins in *P. vulgaris* (Dixon et al.,

1983). From these studies it is apparent that changes in *P. vulgaris* gene expression are initiated prior to condial germination, however, very few studies have focused on the very early changes in gene expression at the whole plant level. Fraire-Velázquez and Lozoya-Gloria (2003), studying the incompatible interaction between *C. lindemuthianum* race 1472 and the cultivar Michigan Dark Red Kidney (MDRK), found that all conidia germinated 12 hours post-inoculation (hpi) and that PAL transcripts accumulated strongly within 2hpi. Additionally, they identified three genes which they suggested are involved in the establishment of the incompatible interaction within 6hpi: beta-glucosidase, Seven in Absentia (*sina*) and SUMO (Small Ubiquitin related MOdifer).

Genome-wide expression analysis is a valuable tool for determining the functions of genes and their spatial and temporal expression patterns, as well as elucidating the genetic networks in which they participate (Schenk et al., 2000). Microarray technology has been implemented to address many questions involving the interaction of plants with various biotic and abiotic stresses. This technology is nevertheless restricted to a small number of organisms for which representative sequence information is available. The cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique provides an alternative approach to microarray analysis and is widely available at a lower cost and does not require prior sequence knowledge (Bachem et al., 1996). The cDNA-AFLP technique is a PCR-based technology similar to differential display, but with higher reproducibility. Specifically, the most critical advantage of cDNA-AFLP over differential display is that cDNA-AFLP is based on linker-ligated PCR whereas differential display is based on PCR which is arbitrarily primed (Bachem et al., 1996).

Anthracnose is a serious seed-borne disease of dry beans. Worldwide, yield losses due to anthracnose are particularly severe in subsistence agricultural systems where beans are a valuable source of plant protein, and income to the producer. Genetic resistance is the most cost effective means to control the disease. Ten major anthracnose resistance genes (*Co-1* – *Co-10*) have been characterized in common bean and are available for use in breeding programs (Kelly and Vallejo, 2004) although none have been cloned. The recently identified allele at the *Co-4* locus, $Co\text{-}4^2$, conditions resistance to 97% of anthracnose races present in North and South America (Balardin and Kelly, 1998; Young et al., 1998) and provides the broadest-based resistance of all of the alleles at the *Co-4* locus (Kelly and Vallejo, 2004). The objectives of this study were to use cDNA-AFLP to (1) determine if any constitutively expressed differences exist between plants which carry the $Co\text{-}4^2$ gene that may contribute to the resistance response, and (2) identify genes which are differentially regulated during the early stages of the incompatible interaction between *C. lindemuthianum* and *P. vulgaris*.

MATERIALS AND METHODS

Plant material and growth conditions. To analyze constitutive differences potentially associated with $Co\text{-}4^2$ mediated resistance, two genotypes which carry $Co\text{-}4^2$ were compared to two which do not carry this gene. These genotypes are referred to as $+Co\text{-}4^2$ and $-Co\text{-}4^2$ respectively. To minimize differences due to genetic background the plant material used was highly related. The two genotypes used that carried $Co\text{-}4^2$ were the breeding line SEL 1308 and a BC_4F_2 progeny, designated A51-2, from the cross

Jaguar*4/SEL 1308. The two genotypes which do not carry the *Co-4*^{2} gene were the black bean cultivar Jaguar and the breeding line SEL 1360. Since Jaguar is the recurrent parent used to develop A51-2, this line is a near-isogenic line from Jaguar. Also, SEL 1360 and SEL 1308 are sister-lines derived from the same backcross with G 2333. Although neither Jaguar nor SEL 1360 carry *Co-4*^{2}, they both are known to carry other anthracnose resistance genes. A51-2 was selected among BC_4F_2 progeny based on the presence of makers linked to the *Co-4* locus: SAS13 (Young et al., 1998) and SBB14 (Awale and Kelly, 2001). SBB14 is a co-dominant marker and was used to select an individual which is not heterozygous at the *Co-4* locus. The selected BC_4F_2 progeny was designated A51-2. Seeds were planted in a growth chamber and provided 12 hours of light. The chamber temperatures were: daytime at 22° C, nighttime at 20°C. Tissue for RNA extraction was collected when the plants had fully expanded primary leaves. Tissues from 6 plants of a single genotype were bulked to eliminate possible single plant effects.

To analyze the pattern of gene expression in the incompatible interaction during the early stages of plant/pathogen interaction, the genotype SEL 1308 was used. Plants were grown in the same conditions as previously described. Tissue was collected at various time points and bulked as described above.

Inoculum preparation and inoculation. Two treatments were used: inoculated (I) and mock-inoculated (MI). In the I treatment, *C. lindemuthianum* race 7 was used. Mock-inoculation was performed using water and 0.01% Tween 20. The fungal isolate were grown from a monosporic culture, maintained on filter papers and stored at -20° C.

Inoculum was prepared by growing the fungus from a filter paper on potato dextrose agar (PDA) at 22°C in the dark. When the cultures began to sporulate, spores were re-plated in replicate to generate sufficient inoculum. Spores were suspended in a solution of water and 0.01% Tween 20. Spore concentration was estimated using a hemacytometer and the concentration was adjusted to 1.2 x 10^6 spores/mL. The prepared inoculum was then sprayed onto the abaxial and adaxial surfaces of the leaves. Inoculated seedlings were incubated at approximately 100% relative humidity for 48 hours. Positive and negative control plants were rated for disease 5 days post inoculation on a scale described by (Balardin et al., 1997). Race classification of the isolate was confirmed prior to use by inoculation on the anthracnose differential series to confirm its identity.

cDNA-AFLP analysis. Total RNA was extracted from bulked tissue samples from time points 0, 0.5, 1, 2, 4, 6, 8, 12, 16, and 20 hpi for both I and MI treated plants. RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Valencia, California). RNA quality and integrity was assessed using electrophoresis and spectrometery. 250 µg of total RNA for each genotype was used for mRNA isolation via the PolyATract™ mRNA System (Promega, Madison, WI). Resulting mRNA samples were speed-vacuumed and resuspended in 10µL of nuclease-free water. Double-stranded cDNA was synthesized from the mRNA samples using the Universal Riboclone cDNA Sysnthesis System (Promega, Madison, WI). cDNA-AFLP analysis was performed as described by Hazen et al. (2002) with the following modifications. No selective bases were used in the pre-selective amplification primers and two selective bases were used in the selective amplification primers. Pre-selective amplification thermal cycler profile used was 2 min

at 94° C; 23 cycles of 1 min at 94° C, 1 min at 56°C, 1 min at 72° C; 5 min at 72° C; 4° soak. The selective amplification thermal cycler profile was: 2 min at 94° C; 12 cycles of 30 s at 94° C, 30 s at 62° C (-0.5°/cycle), 1 min 30 s at 72° C; 29 cycles of 30 s at 94° C, 30 s at 56° C, 45 s at 72° C; 5 min 72° C; 4° C soak. Final PCR products were separated on 6% polyacrylamide gels and visualized with silver staining (Promega, Madison, WI).

Transcript-derived fragment (TDF) cloning and sequence analysis. cDNA-AFLP transcript profiles were analyzed for differences in gene expression. Only fragments larger than 100 bp were considered. In the analysis of constitutive expression differences I focused on bands (referred to as TDFs) which were present in genotypes which have *Co-4*^{2} mediated resistance and absent in those that do not and visa versa. In the analysis of expression differences between I and MI treated plants, I focused on TDFs that were present or absent in two or more consecutive time points. TDFs of interest were cut from fresh gels using a sterile scalpel. The gel slice was then placed in a microcentrifuge tube with 50 µl of water and incubated overnight at 4°C to diffuse the DNA template. Selective PCR was done with the original selective primer combination using 5 µl of the template DNA to re-amplify the fragment. PCR products were then separated on a 2.5 % agarose gel made with 1X TAE and an ethidium bromide concentration of 0.2 mg/mL. TDFs which re-amplified a single product were used for cloning using the Qiagen PCR Cloning Kit (Valencia, California). The cloning reaction was used for the transformation of chemically competent DH5α strain of *E. coli*. The transformation was carried out as suggested in the Qiagen® PCR Cloning Handbook

(April 2001). Sequencing was primed from the T7 or SP6 vector sites. Multiple clones for each TDF were sequenced, trimmed of vector and AFLP adaptor sequences, and aligned using ClustalW (Thompson et al., 1994) to identify a consensus sequence among the clones. Database searches were performed using the BLAST Network Service (NCBI, National Center for Biotechnology Service). Each TDF was compared against all sequences in the non-redundant databases using the BLASTX program, which compares translated nucleotide sequences with protein sequences. TDFs which returned no significant homology were compared again against the EST databases using the BLASTN program. The BLASTP program was used on the six-frame translated amino acid sequence to search for putative protein domains.

RESULTS AND DISCUSSION

Constitutive expression cDNA-AFLP analysis. To gain a better understanding of *Co-4*^{2} mediated anthracnose resistance, I first explored constitutive differences between genotypes which possessed or do not possess the *Co-4*^{2} gene. A total of 149 selective amplification primer pair combinations were tested on the four genotypes: SEL1308, R51-2, SEL1360 and Jaguar. Only 22 TDFs were identified which were clearly polymorphic between the genotypes either carrying or not carrying *Co-4*^{2}. I was only able to obtain sequence information from 14 of the 22 TDFs due to problems with band isolation and purification. It should be noted that multiple sequences of the same size were identified from a single excised band. In these cases, the sequences were analyzed separately. The multiple sequences could represent different transcripts which when restricted with *EcoRI* and *MseI*, produce fragments of the same size. These

fragments would co-migrate in the gel and be re-amplified, post excision, by the same primer combination. Sequence similarity results (Table 3.1) were obtained for nine of the 14 TDFs, although, only six have assigned functions; the remaining TDF sequences were too small to reveal significant homology. The isolated TDFs represent constitutive differences in the expression of specific genes between two genotypes which have *Co-4*^{2} mediated resistance and those that do not. Therefore, these differentially expressed genes may provide some insight into innate differences which exist between resistant and susceptible plants. Patterns of differential expression are summarized in Table 3.1. TDFs C2, C5 and C6.3 were isolated from genotypes which carry the *Co-4*^{2} gene. Based on homology analyses, TDFs C2 and C5, have putative functions in carbohydrate metabolism. TDF C2 is homologous to the Malate dehydrogenase enzyme from *Brassica napus*. Malate metabolism has been implicated as participating in plant defense based on fluctuations in the activities of malate-transforming enzymes (eg. Malate dehydrogenase) and the cellular level of malate upon biotic or abiotic stress. Malate oxidation is carried out by various isoforms of malate dehydrogenase to release reducing equivalents (NADH) and CO_2 (Schaaf et al., 1995). Gross et al. (1977) speculates that a wall-bound malate dehydrogenase may play a role in reactive oxygen species (ROS) production by providing NADH, which is supplied to the cell via a malate oxaloacetate shuttle across the plasma membrane. In lettuce, peroxidase with NAD(P)H oxidase activity are the major source of ROS production (Bestwick et al., 1998). Reactive oxygen species result from the serial reduction of dioxygen. The rapid generation of oxidants has now been described in many plant/pathogen interactions and is a characteristic feature of HR (Allen et al., 1999).

Although cDNA-AFLP is a useful technique for the large-scale analysis of gene expression in plants, this technique unlike microarray analysis, does not address the relative or absolute magnitude of transcript abundance. The cDNA-AFLP technique is useful for identifying the changes or differences of transcript abundance. Therefore, the apparent presence and absence of TDF C2 in the $+Co\text{-}4^2$ and $-Co\text{-}4^2$ genotypes respectively, indicates that those genotypes which carry the $Co\text{-}4^2$ gene have more of the corresponding transcript, which putatively encodes a malate dehydrogenase enzyme, than those genotypes tested which do not posses $Co\text{-}4^2$. Since these plants are capable of mounting $Co\text{-}4^2$ mediated resistance, and they potentially have an increased level of malate dehydrogenase, which is involved in the production of ROS, I can speculate that the elevated level of this enzyme may provide a means for these plants to more quickly produce ROS during an incompatible interaction.

TDF C5 is homologous to the enzyme ADP-glucose pyrophosphorylase (AGPase) from *P. vulgaris*. The key regulatory step in starch biosyntheses is catalyzed by AGPase. Particularly, in leaf and storage tissues in plants, AGPase functions to catalyze the synthesis of ADP-glucose from glucose-1-phosphate and ATP (Muller-Rober et al., 1990). TDF C5 had the same expression pattern as TDF C2 (Table 3.1). Among many metabolic processes, availability of glucose in the cell is important for glycolysis, the process whereby energy which is stored in carbohydrates is released in the form of ATP and NADH. The first step in glycolysis is the breakdown of glucose, a six-carbon sugar, into two three-carbon sugars which are subsequently oxidized and re-arranged to yield two molecules of pyruvate. The apparent increase in ADP-glucose pyrophosphorylase transcript in the $+Co\text{-}4^2$ plants when compared to $-Co\text{-}4^2$ plants, suggests that an increase

in available ADP-glucose and probable higher levels of ATP and NADH, may confer an advantage associated with *Co-4*^{2} mediated resistance. TDF C6.3 is homologous to a putative alpha-mannosidase enzyme from *Zea mays*. The probable function of alpha-mannosidase in plants is in the turnover of glycoproteins and in-the processing of oligosaccharide derivatives, which are implicated in the synthesis of diverse glycoproteins (Cassab and Varner, 1988). Additionally, Boller (1983) suggests that alpha-mannosidase may attack specific wall structures of pathogens. The presence of higher levels of alpha-mannosidase expression in the *+Co-4*^{2} genotypes indicates that there may be a role for this enzyme in defense prior to pathogen attack.

TDFs C7, C17 and C18 represent genes which have higher expression in the genotypes which lack the *Co-4*^{2} gene. TDF C7 is homologous to the plastidic 2-oxoglutarate/malate (malate/OAA) transporter from *Zea mays*. Plastids perform most of the plants biosynthetic reactions. Plastid transporters play an important role in the regulation of metabolism (Dennis and Blakeley, 2000). Additionally, malate is shuttled into the mitochondria via a malate/OAA transporter. Once inside the matrix, malate can be oxidized by two enzymes; malate dehydrogenase, to create reducing equivalents (NADH), or malic enzyme, which generates pyruvates, CO_2 and NADH. It is interesting to note that the expression of the malate dehyrogenase gene (TDF C2) is less in the *–Co-4*^{2} genotypes than in the *+Co-4*^{2} genotypes, thus, if the *-Co-4*^{2} genotypes have increased malate/OAA transporter expression, it may be likely that malate is oxidized more often by malic enzyme than by malate dehydrogenase in the *–Co-4*^{2} plants. Since malate is transported into many different plastids for various processes, it is difficult to speculate on the significance of higher levels of expression in the *–Co-4*^{2} plants, however, this data

may support the importance of metabolic regulation in the ability of a plant to mount the defense response. TDF C17 is homologous to a putative lipoate-protein ligase B which catalyses the formation of an amide linkage between lipoic acid and a specific lysine residue in lipoate dependent enzymes. Lipoylated proteins use this prosthetic group as a carrier of intermediates and reducing equivalents during enzymatic reactions. The major lipoylated proteins are highly conserved and sophisticatedly regulated enzymes of central metabolism (citric acid cycle, TCA), the pyruvate and alpha-ketoglutarate dehydrogenase complexes. The enzymatic activity of these complexes requires the binding of several cofactors including lipoic acid, therefore, the lipoate-protein ligase B, which catalyzes the addition of this cofactor to the complex is important for it's function. It is not obvious what the effect of increased lipoate-protein ligase B (TDF C17) expression is on the activity of these key enzymes complexes of the TCA cycle. However, it is apparent that there are defined differences in gene expression between $+Co\text{-}4^2$ and $-Co\text{-}4^2$ plants at the level of central metabolism. TDF C18 is homologous to translation elongation factor-1 (EF-1) from *Phaseolus coccineus*. Elongation factor-1 is a highly conserved and ubiquitous protein translation factor, therefore, I am unable to speculate on its possible role in defense. However, it should be noted that an elongation factor gene has been mapped to linkage group B7 in *P. vulgaris*. This elongation factor gene is located between the phenylalanine ammonia-lyase-2 gene and the anthracnose resistance gene *Co-6* (Gepts, 1999). There is no evidence, however, that TDF C18 is the same elongation factor which is mapped to this cluster of disease response genes.

Expression pattern of early events in the incompatible interaction. A total of 65 primer pair combinations were tested on cDNA templates prepared from 10 time points of I and MI treatments within a 20 sampling period. These time points were selected to target the earliest changes in gene expression in response to inoculation with an incompatible pathogen. Based on previous studies using various races and bean cultivars, germination of spray inoculated *C. lindemuthianum* conidia occurs within 14hpi (O'Connell et al., 1985; Fraire-Velázquez and Lozoya-Gloria, 2003). Fraire-Velázquez and Lozoya-Gloria (2003) observed single plant cell death and severe conidia degredation at 24hpi of bean plant with an incompatible race. In addition, the fungal structures appeared similar at 48hpi as those observed at 24hpi in the incompatible interaction.

Thirty-four TDFs were identified which appeared differentially expressed between I and MI treatments. Differentially expressed TDFs had to be present or absent in at least two consecutive time points in a treatment to be considered for further analysis. Twenty-two TDFs were successfully re-amplified, cloned and sequenced. The expression patterns of the differentially expressed TDFs are summarized on Table 1.2. Multiple clones per TDF were sequenced and redundancy was eliminated by using CLUSTALW. Multiple sequences from the same TDF were detected in some cases and were treated separately for subsequent analyses. Nine of the 22 TDF sequences had significant similarity with genes of known or putative function (Table 3.2).

TDFs 11.1 and 11.4 represent non-redundant sequences obtained from the same excised band (Figure 3.1). Upon BLASTX analysis, however, they both have similar putative kinase functions. TDF 11.1 is homologous to a putative CRK1 protein from

Oryza sativa. The CRK class of protein is a subclass of the calcium-dependent protein kinase (CDPK) family; CRK stands for CDPK-related kinase (Furumoto et al., 1996). CDRKs are calcium-regulated protein kinases. They contain a single polypeptide which has a catalytic domain for serine/threonine phosphorylation, a junction involved in autoinhibition, and a C-terminal calmodulin (CaM)-like domain with four calcium binding EF hands. CRKs are widely conserved among angiosperms and constitute a small gene family. They, however, are distinct from typical CDPK by their degenerate sequence in the Cam-like domain including four calcium-binding EF-hands. Furumoto et al. (1996) suggest that CRKs actually make up a distinct subfamily of calcium-dependent serine/threonine kinases which may not actually require calcium for their activity. The PlantsP database (http://plantsP.sdsc.edu) reports that most CRKs contain transmembrane domains. In addition, CRKs have a myristoylation motif, necessary for targeting to the plasma membrane (Day et al., 2002). No specific function has been assigned to CRKs in plant cells, however, the expression pattern of TDF 11.1 (Figure 3.1), indicates that this gene is more highly expressed in the I treatment when compared to the MI treatment. This indicates that gene is upregulated in response to the pathogen, starting at 1hpi.

TDF 11.4, isolated from the same band as TDF 11.1, also shows homology to a protein with putative serine/threonine kinase activity from *Oryza sativa.* However, this protein is a receptor serine/threonine kinase (classified as a receptor-like kinase; RLK). Some examples of the role of RLKs in defense are the *Xa21* gene in rice and the *Pto* gene of tomato. The *Xa21* gene, which conditions resistance to bacterial leaf light (*Xanthomonas oryzae* pv. *oryzae*), encodes a membrane-bound RLK with an extracellular leucine-rich repeat (LRR). Although no ligand is known which binds with this gene

product, the presence of the extracellular LRR suggests a receptor role in pathogen recognition (Hardie, 1999). The *Pto* gene of tomato, which confers strain-specific resistance to *Pseudomonas syringae*, encodes a serine/threonine protein kinase with N-terminal myrostoylation sites, but has no extracellular or transmembrane domains. To condition resistance, *Pto* requires the presence of *Prf*, which encodes a LRR containing protein. The Pto gene is classified as a RLK due to the similarity at the kinase domain (Hardie, 1999). The upregulation of TDF 11.4 in response to inoculation with *C. lindemuthianum* suggest that this receptor serine/threonine kinase may be involved in perception of the pathogen. The only known resistance gene whose expression is induced by inoculation is the *Xa1* gene in rice which conditions resistance *Xanthomonas oryzae* pv. *oryzae* but does not contain a kinase domain (Yoshimura et al., 1998).

Since both TDF 11.1 and 11.4 were isolated from the same band, and the homology analysis revealed that they are similar to two different putative proteins, it is not possible to conclusively say that the expression pattern observed by cDNA-AFLP corresponds to both. To confirm that these two TDFs are upregulated in response to inoculation, northern analysis using each TDF as a separate probe is necessary. Because the TDF 11.1 and 11.4 sequences are so small, only 156 and 167 bases respectively, and both TDFs encode putative serine/threonine kinase proteins, it is possible that they are two pieces of the same gene; perhaps each represents different domains of the same protein. An additional caveat is the ubiquitous nature of kinases. Kinase proteins are involved in the regulation of many cellular processes such as cell division, metabolism and responses to biotic and abiotic stresses and approximately 4% of *Arabidopsis* genes encode for typical kinase proteins (Hrabak et al., 2003).

TDF 12 encodes for a beta-mannosidase. This enzyme belongs to the family 1 glycosidases. Among this family of enzymes, the beta-glucosidase protein has been previously reported to be upregulated in response to a host/pathogen interaction in *Z. mays/B. maydis* (Simmons et al., 2001) and in *P. vulgaris/C. lindemuthianum* (Fraire-Velázquez and Lozoya-Gloria, 2003). In both of these studies the beta-glucosidase gene was suggested to have a role in the defense response although no biochemical evidence is presented. However, Hsieh and Graham (2001) suggest that beta-glucosidase is involved in the release of two isoflavone-conjugated compounds which have a central role in the defense response in *Glycine max.* Differential expression of the beta-glucosidase gene identified by Fraire-Velázquez and Lozoya-Gloria (2003) was detected between 1 and 6 hpi of intact bean plants, spray inoculated with an incompatible race of *C. lindemuthianum.* Because they bulked the tissue samples from time points 1h – 6hpi for the subtractive hybridization procedure, I was unable to determine the first time point of detection and that of greatest accumulation. Despite this, the expression of TDF 12 detected between time points 6 through 12 hpi does not contradict these finding. Therefore, I believe that the beta-mannosidase, also a family 1 glycosyl hydrolase, identified in the current study is under similar regulation as the beta-glucosidase detected by Fraire-Velázquez and Lozoya-Gloria (2003) and may also have a role in the defense response. Fraire-Velázquez and Lozoya-Gloria observed that cytological differences were obvious in pathogen development between the incompatible and compatible interactions after only 14 hpi. The authors used the bean cultivar Michigan Dark Red Kidney, known to carry the *Co-1* locus for anthracnose resistance. The fungal conidia in the incompatible interaction were severely damaged by 24 hpi and they observed fungal

cell debris around the collapsed and aggregated spores. Dead single plant cells were observed at 24 hpi in the incompatible interaction, indicating HR. Fraire-Velázquez and Lozoya-Gloria suggest that the beta-glucosidase activity could have contributed to the severe degredation of the conidia.

The expression pattern of TDFs 18.1, 18.2, 22 and 23 (Figure 3.1) are strikingly similar in that transcript accumulation increased between time points 1h and 6hpi, decreased between 8h and 12hpi and increased again between 16h and 20hpi. A very similar temporal accumulation pattern was reported for the L-phenylalanine ammonia-lyase (PAL) gene in the incompatible interaction between *P. vulgaris* and *C. lindemuthianum.* PAL is a key enzyme in the production of phytoalexins in *P. vulgaris* through the phenylpropanoid biosynthetic pathway (Fraire-Velázquez and Lozoya-Gloria, 2003). Phytoalexins are low molecular weight, lipophilic, antimicrobial compounds that accumulate rapidly around sites of the incompatible pathogen infection (Smith, 1996). This would suggest that the TDFs which I identified, are regulated similarly to PAL, and therefore, may play a role in the response of *P. vulgaris* to an incompatible race of *C. lindemuthianum.* TDF 12, may also be similarly regulated since its pattern of accumulation is directly opposite that of TDFs 18.1, 18.2, 22 and 23 (Table 3.2) , and it too seems to be related to a gene involved in the defense response of *P. vulgaris* to incompatible races of *C. lindemuthianum.*

The expression of TDFs 18.1 and 18.2 in the MI treated plant appears to be constitutive, however, it should be noted that time point 0h in the MI treated plants is more intense than that of time point 0h of the I treated plants. The expression of this time point between the two treatments should be the same, therefore, this observed difference

may represent an increased amount of RNA used in the initial steps of the cDNA-AFLP analysis. TDF 18.1 is homologous to a putative lysine decarboxylase-like protein from *Oryza sativa* (Table 3.2). BLASTP analysis revealed that TDF 18.1 contains a lysine decarboxylase domain (Figure 3.2). Lysine decarboxylase is present in some bacteria, but also several higher plants, particularly Graminae, Leguminoseae and Solanaceae. The function of the lysine decarboxylase enzyme is in the catalysis reaction of L-lysine to cadaverine and CO_2 (Crozier et al., 2000). Cadaverine is a polyamine, which is a low molecular mass polycation that has antiherbivory properties and has also been implicated in molecular signaling events in plant pathogen interactions (Martin-Tanguy, 2001). The activity of cadaverine is positively correlated with the accumulation of quinolizidine alkaloids in the aerial parts of the lupin plant where their defensive properties are most effective (Wink and Hartmann, 1982). Additionally, lysine decarboxylase activity was found to be positively correlated with chlorophyll levels (Martin-Tanguy, 2001). The conjugation of polyamines to phenolics has often been described as a defense mechanism against infection of several higher plants by viruses and fungi (Fleurence and Negrel, 1989; Louis and Negrel, 1991). Therefore, the lysine decarboxylase enzyme may play a role in the early response of *P. vulgaris* to *C. lindemuthianum* in the incompatible interaction.

TDF 18.2 encodes a NIM1-like protein 1 from *Helianthus annuus*. NIM1, stands for Non-inducible IMunity1 (Delaney et al., 1995), and is also called NPR1, which stands for Non-expressor of Pathogenesis-Related genes1 (Cao et al., 1994). The transduction of the salicylic acid (SA) signal to activate the production of pathogenesis-related (PR) proteins, requires the function of NIM1. It is a regulatory protein that was identified

through screening systemic-acquired resistance compromised mutants of *Arabidopsis* (Delaney, 1997). In addition to local defense responses such as HR, recognition of avirulent pathogens can trigger a secondary defense response which gives the plant a long-lasting, broad-spectrum disease resistance throughout the entire plant which is referred to as systemic-acquired resistance (SAR) and is accompanied by the production of PR proteins (Ryals et al., 1996). NIM1 expression in plants is constitutive, however, upon induction of SAR, it is translocated to the nucleus to act as a modulator of PR gene expression but does not bind DNA directly. NIM1 enhances the DNA binding of a basic leucine zipper (bZIP) family of transcription factors, referred to as TGAs, to the SA-responsive promoter elements in the *Arabidopsis PR-1* gene. The NIM1 gene contains both a BTB/POZ domain and ankyrin repeats (Cao et al., 1997). BLASTP analysis revealed that TDF 18.2 contains a BTB domain (Figure 3.2). The BTB/POZ domain is an evolutionarily conserved region of about 120 amino acids, originally named for the *Drosophila* Broad-Complex, Tramtrack and Bric-a-brac proteins (BTB) (Zollman et al., 1994), in which the Pox virus and Zinc finger proteins can be found, (Bardwell and Treisman, 1994). Ankyrin repeats are tandemly repeated amino acid modules of about 33 residues, and are considered the most common protein interaction motif (Sedgwick and Smerdon, 1999). The expression pattern of TDF 18.2, a NIM1-like gene, suggests a possible role for that gene in the regulation of genes involved in the defense response.

TDFs 22 and 23 both encode enzymes involved in the biosynthesis of chlorophyll. Their pattern of expression, which is the same as that for TDFs 18.1 and 18.2, may at first appear diurnally regulated; however, the constitutive expression of these genes in the MI treated plants suggests that the change in transcript accumulation is not the result of the

20h sampling period of this study. Although there is not an obvious link for these proteins in the defense response, similar results have been reported in other studies which suggest crosstalk between the light and defense signal transduction pathways. In the microarray analysis of the defense response of *Arabidopsis*, Schenk et al. (2000) found that SA treatment induces the expression of genes encoding chlorophyll a/b-binding proteins (CABs). In contrast, they found that the application of methyl jasmonate down-regulated the gene for ribulose bisphosphate carboxylase. It should be noted that the RNA samples for this study were taken at 24 hours post chemical treatment. Genoud and Metraux (1999) showed that the *Arabidopsis* phytochrome A and B signaling mutant, *psi2*, had elevated levels of PR gene expression and suggested crosstalk between the light signal transduction and PR gene signaling pathways.

TDF 31, which encodes a zinc finger (C3H-type) family protein from *Arabidopsis*, has a unique expression pattern among the TDFs reported in this study. TDF 31 expression appears generally repressed in the I treated plants and expressed in the MI plants. Plants from both treatments at time point 0hpi have expression of the gene. This gene is quickly down-regulated in both the I and MI treated plants, however, this gene remains repressed in the I treated plants, whereas, expression is increased again in the MI treated plants between 2h and 4hpi. Expression is not detected at time point 6hpi in the MI treated plants, but then increases between 8h and 20hpi. The zinc finger motif is involved in protein binding to DNA as well as protein-protein interaction. They are classified into C2H2, C3H, C4 and ring finger types based on the amino acid sequences comprising the zinc finger domain (Garrett and Grisham, 2002). In a microarray experiment addressing the analysis of gene expression during the defense

response in *Arabidopsis*, Schenk et al., (2000) found that the treatment of plants with SA or methyl jasmonate down-regulated the expression for a zinc-finger transcription factor. They also found that inoculation with an incompatible fungus and treatment with ethylene also down-regulated two zinc-finger proteins. The zinc-finger protein from *Arabidopsis*, LSD1 (lesion-simulating disease resistance), is a negative regulator of the hypersensitive response (Dietrich et al., 1997), therefore, the involvement of genes encoding transcription factors with zinc-finger/binding domains in regulating plant defense responses appears very likely. The repressed expression of TDF 31 supports a putative role as a negative regulator of the defense response.

Comparison of constitutive and induced differences in plants which can undergo R-gene mediated resistance. All of the TDFs which were identified in the analysis of constitutive differences were neither induced nor repressed in response to inoculation with an incompatible race of *C. lindemuthianum*. Despite the fact that they are not inducible does not signify that they are not important for the defense response to take place. These differences represent innate differences in basic metabolism which may provide an advantage or a disadvantage in a plants ability to more quickly trigger the defense response. The TDFs isolated from the analysis of the incompatible interaction were not differentially expressed between $-Co\text{-}4^2$ and $+Co\text{-}4^2$ plants in the study of constitutive expression differences, supporting the idea that these TDFs represent an active response in the plant to alter gene expression.

CONCLUSIONS

The cDNA-AFLP technique enabled the discovery of genes which may be involved in the defense response at the constitutive and induced expression levels. Eight genes were identified with putative protein functions previously associated with various aspects of the defense response. I was particularly interested in identifying genes which play a critical role in the early stages of the plant/pathogen interaction which may be determinants for the final response of the plant. In the study of the incompatible interaction, changes were observed in gene expression as early as 1hpi suggesting that these changes occur possibly earlier than pathogen recognition, assumed to occur after penetration of the fungus into the plant cell. The genes identified may represent genes which are regulated by the interaction or possible regulators of downstream genes as in the case with the zinc-finger protein. However, the expression pattern of the identified genes must be confirmed by either Northern analysis or by real-time PCR to validate these results. Determining the genomic location of these genes on the bean integrated linkage map (Freyre et al., 1998), and their relative proximity to known anthracnose resistance loci, may provide linked molecular markers for use in the marker-assisted breeding of anthracnose resistant bean cultivars. The demonstration that specific changes in gene expression occur at an early stage in the plant defense mechanism, may lead to a better understanding of signal-response coupling mechanisms in plant-pathogen interactions, and the development strategies to manipulate and enhance the response of plant cells to biological stress (Schenk et al., 2000).

Table 3.1 Analyses of TDF sequence homology using BLASTX and BLASTN and their expression pattern from constitutive experiment.

TDF	Length (bp)	Accession no.	Putative Identity	Pattern of gene gene expression[a]	E value
C2	143	gi|4995089	Malate dehyrogenase 1 (*B. napus*)[b]	+*Co-4*^{2}	$1e^{-18}$
C5	235	gi|29421115	ADP-glucose pyrophosphorylase (*P. vulgaris*)[c]	+*Co-4*^{2}	$7e^{-41}$
C6.3	188	gi|37530956	Putative alpha-mannosidase (*O. sativa*)[b]	+*Co-4*^{2}	$6e^{-20}$
C7	197	gi|40363459	Plastidic 2-oxoglutarate/Malate transporter (*Z. mays*)[c]	-*Co-4*^{2}	$1e^{-8}$
C17	125	gi|27387702	Probable lipoate-protein Ligase B (*P. coccineus*)[d]	-*Co-4*^{2}	$2e^{-51}$
C18	150	gi|27392436	Translation elongation factor-1 (*P. coccineus*)[c]	-*Co-4*^{2}	$8e^{-13}$

[a] +*Co-4*^{2} signifies the band was present in genotypes carrying *Co-4*^{2} ; -*Co-4*^{2} signifies present in genotypes without *Co-4*^{2}.

[b] Homology analyses of TDF sequences using BLASTX

[c] Homology analyses of TDF sequences using TBLASTX

[d] Homology analyses of TDF sequences using BLASTn

Table 3.2 Analyses of TDF sequence homology using BLASTX and their expression pattern from cDNA-AFLP analysis of inoculated and mock-inoculated plants. TDFs 11.1 and 11.4 were isolated from the same band. TDFs 18.1 and 18.2 were also isolated from the same band.

TDF	Length (bp)	Accession no.	Putative Identity gene expression	Pattern of gene[a]	E value
11.1	156	gi|34903662	Putative CRK1 protein (*O. sativa*)	↑ 1h-20hpi	$2e^{-20}$
11.4	167	gi|34901978	Putative receptor ser/thr protein (*O. sativa*)		$1e^{-13}$
12	94	gb|AAL37714.1	Beta-Mannosidase enzyme (*L. esculentum*)	↓ 1h-4, ↑ 6h-12, ↓ 16h-20	$6e^{-6}$
18.1	420	gi|50916205	Putative lysine decarboxylase-like protein (*O. sativa*)	↑ 2h-6, ↓12h-16, ↑ 20hpi	$6e^{-49}$
18.2	410	gi|49182284	NIM1-like protein1 (*H. annuus*)		$2e^{-26}$
22	132	gi|25299023	Chlorophyll a/b-binding protein Lhca5, PS I (*A. thaliana*)	↑ 2h-4, ↓ 6-16h, ↑20h	$1e^{-14}$
23.1	172	sp|P09756	Chlorophyll a/b-binding protein 3, chloroplast precursor (*G. max*)	↑ 2h-6, ↓ 8h-16, ↑ 16h-20	$5e^{-26}$
31	165	gi|18395319	Zn finger (C3H-type) family protein (A. thaliana)	↓ 0.5h-20hpi	$5e^{-5}$

[a] expression pattern is summarized using arrows: ↑ indicates increase in expression, ↓ indicates decrease in expression

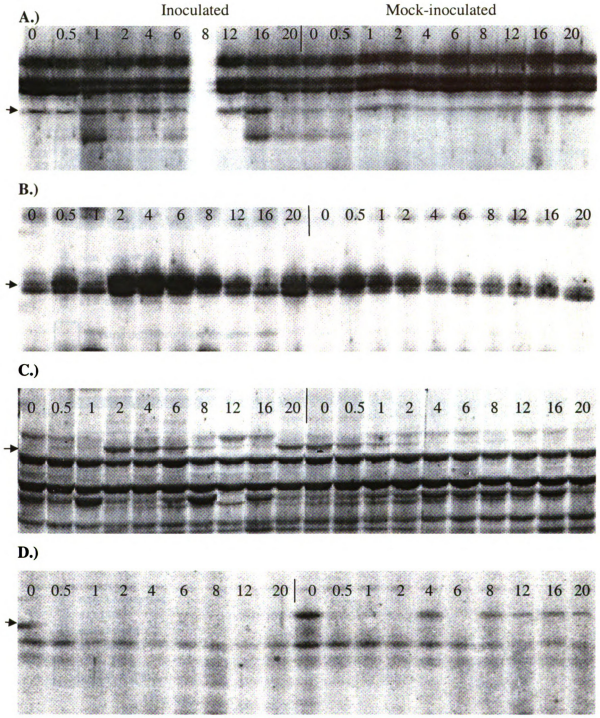

Figure 3.1 cDNA-AFLP expression profiles across time points 0, 0.5, 1, 2, 4, 6, 8, 12, 16 and 20 hpi for inoculated and mock-inoculated treatments. **(A)** TDF 11.1 and 11.4 **(B)** TDF 18.1 and 18.2 **(C)** TDF 22 and 23, although TDFs 22 and 23 were isolated from different bands, their expression pattern is identical **(D)** TDF 31, note that time point 16h is missing.

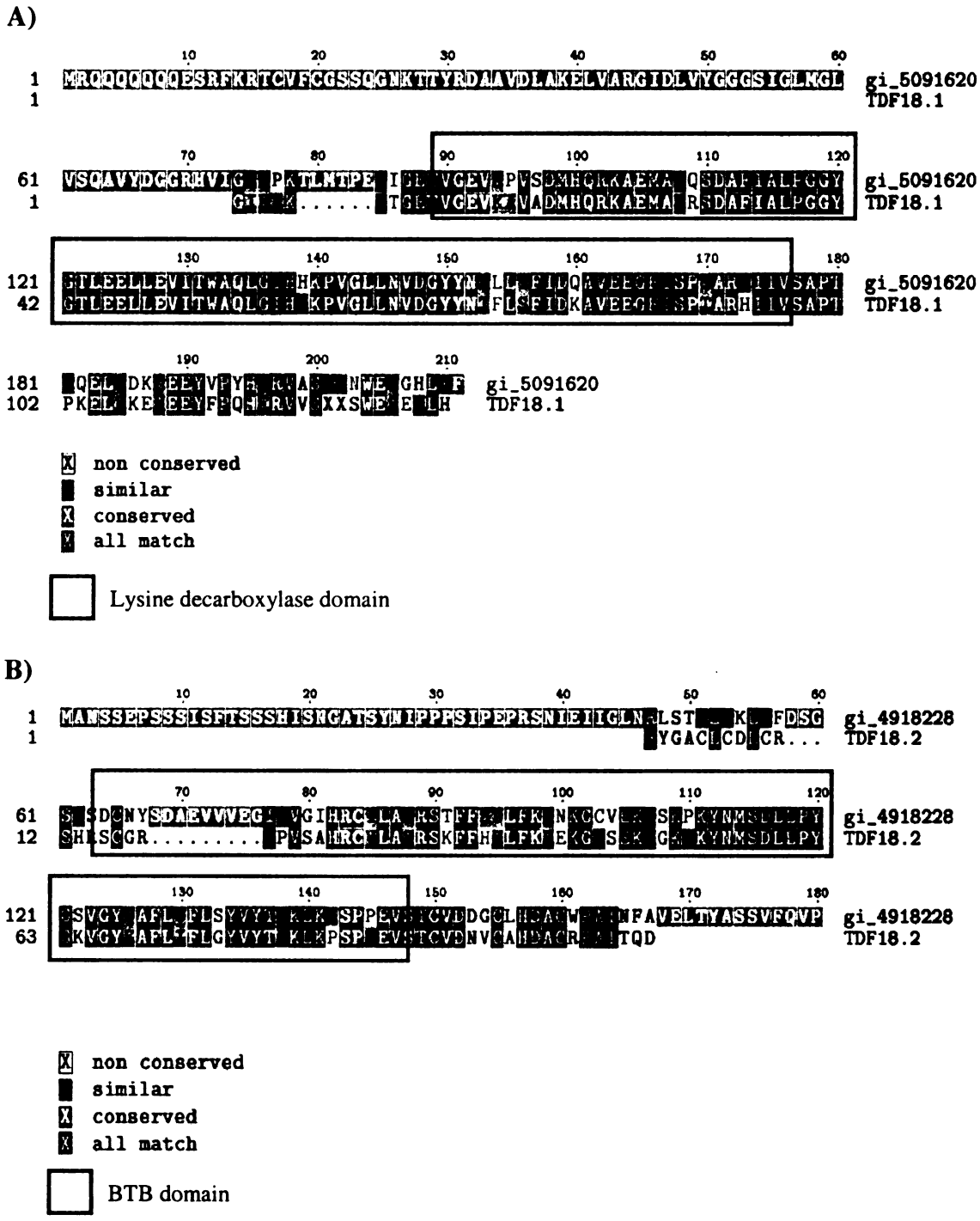

Figure 3.2 Sequence alignment generated by ClustalW. Putative protein domains detected by the BLASTP program are boxed. **A)** Alignment with gi|50916205 and TDF 18.1 **B)** Alignment with gi|49182284 and TDF 18.2.

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APPENDICES

APPENDIX A1

INITIAL DISSECTION OF THE ANTHRACNOSE RESISTANCE IN THE LANDRACE CULTIVAR G 2338

INTRODUCTION

Anthracnose, is a seedborne disease of common bean (*Phaseolus vulgaris*), caused by the hemibiotrophic fungus, *Colletotrichum lindemuthianum*. The devastating effect of anthracnose on yield, particularly in tropical production areas, makes it one of the most economically important diseases of bean. Several disease management strategies are available, however, planting genetically resistant cultivars is the most effective and least expensive way for growers to prevent yield loss due to anthracnose (Pastor-Corrales et al., 1995). The best breeding strategy to generate resistant cultivars is by pyramiding anthracnose resistance genes with complementary spectra of resistance (Kelly and Vallejo, 2004). The highly variable nature of the pathogen, however, requires that new sources of resistance are constantly being characterized. The landrace cultivar G 2333, from Chipas, Mexico, is the most resistant cultivar of the anthracnose differential series and carries three independent resistance genes (*Co-4*^{2}, *Co-5* and *Co-7*). Recently, a highly virulent *C. lindemuthianum* race of Middle American origin (Costa Rica) has been identified which overcomes the resistance in G 2333. Interestingly, the landrace G 2338, also from Chiapas, Mexico is resistant to this race (J. Kelly personal communication). Since both landraces are from the same region of Mexico, we hypothesize that the

differential resistance is conferred by a small difference in their allelic composition, therefore, we postulate that G 2338 carries a similar anthracnose gene pyramid. The objective of this study was to initialize the molecular and genetic characterization and dissection of the anthracnose resistance genes carried by G 2338.

MATERIALS AND METHODS

Initial characterization of the anthracnose resistance in G 2338 was carried out using molecular markers linked to known anthracnose resistance genes (Table A1.1). Additionally, G 2338 was previously tested for the presence of several SCAR markers linked to the *Co-4* locus and the *Co-4*^{2} allele (Table A1.2) (Awale and Kelly, 2001). The number of genes conditioning resistance to anthracnose in G 2338 was determined by inoculating a F_2 population of 201 individuals from the cross La Victorie (susceptible) x G 2338 (resistant) with race 7. Based on the results of the initial characterization with molecular markers linked to anthracnose resistance genes, a SCAR marker was used to begin the molecular dissection of the gene pyramid in G 2338.

RESULTS AND DISSCUSSION

The initial characterization of the anthracnose resistance genes carried by G 2338, based on analysis with molecular markers linked to various known genes, indicated that G 2338 carries the *Co-5* and *Co-4* loci (Table A1.1). Since allele specific primers exist for the *Co-4* locus, we wanted to determine if the G 2338 carries the same allele as G

2333 at the *Co-4* locus. The results displayed in Table A1.2 indicate that G 2338 does not carry the same allele at the *Co-4* locus as G 2333. To determine the number of genes segregating for resistance to anthracnose a F_2 population (La Victorie x G 2338) was inoculated with race 7 which yields a SxR reaction in the parents. Out of the 201 F_2 individuals inoculated, 9 were lost to lethal combinations of dwarfing genes due to the intergene pool nature of the cross. The remaining progeny segregated in a 63:1 ratio of resistant to susceptible indicating that three genes are segregating for resistance to race 7 (p-value= 0.244). Based on our preliminary gene characterization using molecular markers we believe that two of the genes are *Co-5* and *Co-4*. To begin the molecular dissection of this gene pyramid, we used the SCAR marker SAB3 linked to the *Co-5* locus to select against this locus among the surviving progeny of the F2 La Victorie x G 2338 population. Unfortunately, we were not able to elucidate the identity of the third gene conditioning resistance to anthracnose in G 2338. However, based on the similar origin of both of these landraces and the fact that they both have three genes conditioning resistance to anthracnose two of which are the same, we speculate that the third gene in G 2338 is very likely to be *Co-7*. Given the allele complexity at the *Co-4* locus we believe that the resistance differential between G 2333 and G 2338 is due to differences in the allelic composition at the *Co-4* locus.

Table A1.1 RAPD markers linked to anthracnose resistance genes

Molecular marker	Linked resistance gene	Linkage phase	+/-[a]
$F10_{530}$	*Co-1*	repulsion	-
$Q4_{1440}$	*Co-2*	coupling	-
$H20_{500}$	*Co-2*	coupling	-
$AB3_{400}$	*Co-5*	coupling	+
$AH1_{780}$	*Co-6*	coupling	-
$AK20_{890}$	*Co-6*	repulsion	-
$AS13_{950}$	*Co-4*	coupling	+
SB12	*Co-9*	coupling	-

[a] Presence (+) or absence (-) in G 2338

Table A1.2 SCAR markers linked to the *Co-4* locus or specific alleles.

SCAR marker	Locus	G 23333	G 2338
SH18	*Co-4*^{2}	+	-
SAS13	*Co-4*	+	+
SBB14	*Co-4*	+	+

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APPENDIX A2

UNEXPECTED RESISTANCE GENES UNCOVERED

Genetic improvement in dry bean is achieved through a combination of traditional and molecular selection techniques. The resulting cultivars are pure lines which are improved for one or more specific traits. Despite the homozygous nature of bean cultivars, sources of genetic variation may be mutations, insect cross pollination or the cultivars' heterogeneity (Traka-Mavrona et al., 2000). In the absence of direct selection, many pure-line cultivars may be heterogeneous for major resistance genes. The following are case studies in which new resistance genes were unexpectedly uncovered in pure line cultivars.

Black Magic

The black bean cultivar, Black Magic (BM), was reported to carry resistance to races beta, gamma and delta of *C. lindemuthianum* (Kelly et al., 1987). In the currently used nomenclature for race classification, these are races 130, 102 and 23 respectively (Melotto et al., 2000). Black Magic, however, unexpectedly segregated for resistance to race 7 (Melotto, 1999). Although BM carries resistance to anthracnose races 23, 102 and 130, anthracnose resistance was not a specific goal in cultivar development (Kelly, personal communication). Our hypothesis is that the resistance to race 7 in BM is

conferred by a single dominant gene. To determine the number of genes conferring resistance to race 7 in BM a F_2 population was generated from the cross BM (resistant to race 7; R7) x BM (susceptible to race 7; S7). The resulting population was inoculated with race 7 (Table A2.1). The F_2 population, BM (R7) x BM (S7), segregated in a 15:1 ratio of resistant to susceptible individuals (p-value = 0.936) (Table A2.1) indicating that the resistance to race 7, in the cultivar BM, is conditioned by two dominant resistance genes. Allelism tests must next be carried out to determine if these genes are independent of the other previously described anthracnose resistance genes. We believe that this unexpected segregating resistance to anthracnose is a result of the cultivars natural heterogeneity which exists for specific traits which are not under direct selection during cultivar development.

Mexico 222

The anthracnose differential cultivar Mexico 222 is believed to carry a single dominant gene, *Co-3* which conditions resistance to anthracnose (Kelly and Vallejo, 2004). To determine if the anthracnose resistance in the MSU breeding line, MSU7-4, was allelic to *Co-3*, a F_2 population was generated from the cross Mexico 222 x MSU 7-4. The resulting population was inoculated with race 7, which results in a RxR reaction in the parents (Table A2.1). The Mexico 222 x MSU7-4 F_2 population segregated for resistance at a ratio of 63:1 (p-value = 0.979), resistant to susceptible individuals. This indicates that there are three genes segregating for resistance to race 7 in this population. Since race 7 elicits a RxR reaction in the parents (Table A2.1), and it was previously determined that MSU7-4 carries only one gene conditioning resistance to race 7 (chapter

two of this dissertation), we conclude that two anthracnose resistance genes must be segregating from the cultivar Mexico 222. One of the anthracnose resistance genes in Mexico 222 is known to be *Co-3* and the other remains unknown. Neither of these anthracnose resistance genes are allelic to the gene carried by MSU7-4. Heterogeneity within the cultivar can be used to explain this unexpected discovery of an additional anthracnose resistance gene in Mexico 222. Alternatively, with the abundance of different *C. lindemuthianum* races available, and the implicit gene-for-gene relationship between resistance in the plant and pathogenicity in the pathogen, it is not surprising if some anthracnose resistance genes go undetected in certain population/race inoculation experiments. Additional characterization of the second anthracnose resistance gene in Mexico 222 is needed to determine if it is independent of the previously reported anthracnose genes and what spectrum of resistance it offers.

Table A2.1 F2 populations inoculated with race 7 of *C. lindemuthianum*.

Population or Genotypes	Race Inoculated	Disease Reaction	Observed Ratio[a]	Expected Ratio[a]	p-value
BM (R7) x BM (S7)[b]	7	-	87:6	15:1	0.936
Mexico 222 x MSU7-4	7	-	249:4	63:1	0.979
Mexico 222	7	R	-	-	-
MSU7	7	R	-	-	-

[a] Ratio of resistant to susceptible individuals

[b] R7 = resistant to race 7, S7= susceptible to race 7

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