

Identification of an Ancestral Resistance Gene Cluster Involved in the Coevolution Process Between *Phaseolus vulgaris* and Its Fungal Pathogen *Colletotrichum lindemuthianum*

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The recent cloning of plant resistance (R) genes and the sequencing of resistance gene clusters have shed light on the molecular evolution of R genes. However, up to now, no attempt has been made to correlate this molecular evolution with the host-pathogen coevolution process at the population level. Cross-inoculations were carried out between 26 strains of the fungal pathogen *Colletotrichum lindemuthianum* and 48 *Phaseolus vulgaris* plants collected in the three centers of diversity of the host species. A high level of diversity for resistance against the pathogen was revealed. Most of the resistance specificities were overcome in sympatric situations, indicating an adaptation of the pathogen to the local host. In contrast, plants were generally resistant to allopatric strains, suggesting that R genes that were efficient against exotic strains but had been overcome locally were maintained in the plant genome. These results indicated that coevolution processes between the two protagonists led to a differentiation for resistance in the three centers of diversity of the host. To improve our understanding of the molecular evolution of these different specificities, a recombinant inbred (RI) population derived from two representative genotypes of the Andean (JaloEEP558) and Mesoamerican (BAT93) gene pools was used to map anthracnose specificities. A gene cluster comprising both Andean (*Co-y*; *Co-z*) and Mesoamerican (*Co-9*) host resistance specificities was identified, suggesting that this locus existed prior to the separation of the two major gene pools of *P. vulgaris*. Molecular analysis revealed a high level of complexity at this locus. It harbors 11 restriction fragment length polymor-

phisms when R gene analog (RGA) clones are used. The relationship between the coevolution process and diversification of resistance specificities at resistance gene clusters is discussed.

Additional keywords: common bean, mapping.

Plant disease resistance often results from the presence of a specific resistance (R) gene in the plant and a corresponding avirulence gene in the pathogen. R genes obeying this gene-for-gene (GFG) model (Flor 1971) have been identified in cultivated as well as wild plants (Barrett 1985; Burdon 1994; Clarke 1997). A high level of diversity for resistance was found in natural plant populations (Burdon 1996) but the mechanisms maintaining this diversity are still not clearly understood. The process of coevolution resulting from the reciprocal selection pressure exerted by the plant and the pathogen is widely considered to be a potential mechanism acting on resistance and virulence diversity in wild populations (Thompson and Burdon 1992; Simms 1996; Thrall and Burdon 1997). However, despite many theoretical studies (Burdon 1996; Frank 1992, 1997; Kaltz and Shykoff 1998), few biological experiments have been performed to investigate the GFG coevolution between plant and pathogen (Thompson and Burdon 1992). Two approaches have been mainly used to analyze the coevolution process at the biological level. The first approach compares the population structure of both plant and pathogen (Burdon and Jarosz 1991; Burdon and Thompson 1995; Jarosz and Burdon 1991). When the organization of genetic variation does not match between the two protagonists, stochastic forces rather than coevolution are supposed to be responsible for the maintenance of resistance and virulence diversity. This has been strongly suggested for the interaction between flax (*Linum marginale*) and the rust fungus (*Melampsora lini*) in which diversity was supposed to result from extinction/recolonization events rather than selection (Burdon and Jarosz 1991; Burdon and Thompson 1995; Jarosz and Burdon 1991). In contrast, it has been commonly argued that a parallelism in the

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population structure for both plant and pathogen is an indicator of coevolution processes. However, the same population structure for the two protagonists does not provide undisputed evidence of coevolution because it can be the result of a similar migration/drift dynamic that may have affected both partners. A second approach, more direct and complete, compares the performance of the pathogen on sympatric and allopatric hosts. Studies carried out on various types of interactions, including plant/pathogen, plant/herbivore, invertebrate/microparasite, and vertebrate/ parasite interactions (reviewed in Kaltz and Shykoff 1998), showed independently that the coevolution process often resulted in an adaptation of the pathogen to a host of the same origin, leading to a differentiation of the host populations for resistance. However, the genomic organization of the genes underlying the coevolution process was never considered in these analyses.

Genetic and molecular studies have been carried out to investigate the evolution of plant R genes (Ronald 1998). Genetic studies 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). Complex resistance loci reported in corn (Richter et al. 1995), flax (Ellis et al. 1995), lettuce (Witsenboer et al. 1995), or barley (Jorgensen 1992) clearly obeyed this rule. Furthermore, clustering of R genes conferring resistance to diverse pathogens was also reported, as for instance on the short arm of chromosome 6 in tomato (Dickinson et al. 1993; Jorgensen 1992; Kaloshian et al. 1995; Vanderbeek et al. 1994; Zamir et al. 1994).

Several R genes derived from various plant species and active against diverse pathogens have now been cloned (Hammond-Kosack and Jones 1997). These genes appeared to belong to a restricted number of classes, the prevalent class encoding nucleotide binding site-leucine rich repeat (NBS-LRR) containing proteins (Hammond-Kosack and Jones 1997). More recently, a large number of R gene analogs (RGAs) were characterized with degenerate oligonucleotide primers matching conserved sequences from R genes cloned in diverse plant species. Many of these RGAs are arranged in clusters and are co-localized with known resistance specificities (Aarts et al. 1998; Botella et al. 1997; Kanazin et al. 1996; Leister et al. 1996, 1998; Shen et al. 1998; Speulman et al. 1998; Yu et al. 1996). Phylogenetic analyses of RGA sequences from different plant species strongly suggested that RGA sequences existed in the plant genome prior to species divergence (Michelmore and Meyers 1998). Classical genetic analysis in maize (Richter et al. 1995; Sudupak et al. 1993) as well as sequencing of several R gene clusters in rice (Song et al. 1997), tomato (Parniske et al. 1997), and lettuce (Meyers et al. 1998), revealed that point mutations, unequal crossing-overs, gene conversions, and transpositions have led to the high diversity observed at R gene clusters.

The regions of R genes responsible for resistance-specific recognition of a pathogen are still not completely understood. Recent data indicate that the sequence encoding solvent-exposed residues within the beta-strand/beta-turn structural motif of LRR proteins might be involved in the specificity of ligand binding (Parniske et al. 1997; Wang et al. 1998). Although data are now available on the molecular mechanisms leading to new R genes, the relationship between these mo-

lecular changes and the evolutionary forces maintaining some of these resistance specificities in the genome remains a matter of conjecture.

Until now, GFG coevolution processes and molecular evolution of R gene clusters have been studied independently and no attempt has been made to correlate these two aspects of plant/pathogen interactions. In this regard, the interaction between common bean, *Phaseolus vulgaris*, and its fungal pathogen *Colletotrichum lindemuthianum*, the causal agent of anthracnose, is especially suitable for analysis since (i) diversity of wild and cultivated common bean has been well documented (Gepts and Debouck 1991), (ii) diversity of *C. lindemuthianum*, a seed-borne pathogen of common bean with a low dissemination rate (Tu 1992), has been studied in wild populations of its host (Sicard et al. 1997), and (iii) a dense map of common bean is available (Freyre et al. 1998). Based upon morphological traits (Delgado-Salinas et al. 1988), phaseolin electrophoretic types (Gepts 1988; Kami et al. 1995), isozymes (Debouck et al. 1993; Koenig and Gepts 1989; Singh et al. 1991), and restriction fragment length polymorphisms (RFLPs; Becerra-Velásquez and Gepts 1994; Sonnante et al. 1994), three areas of diversity can be distinguished for *P. vulgaris* natural populations: the Mesoamerican, the South Andean, and the North Andean centers. The associated *C. lindemuthianum* populations from Mexico, Ecuador, and Argentina (in the Mesoamerican, North Andean, and South Andean centers of diversity, respectively) were found to be highly differentiated for neutral markers and for virulence on cultivated common beans, suggesting existing mechanisms of coevolution and/or similar migration/drift dynamics (Sicard et al. 1997). Genetic analyses based on neutral markers provide evidence for two independent domestication processes within the South Andean and Mesoamerican centers of diversity, leading to the development of two distinct groups of cultivated beans, called the Andean and Mesoamerican gene pools (Gepts 1993, 1998; Gepts and Debouck 1991), respectively.

Three goals were pursued in the present study. First, a cross-inoculation experiment between wild *P. vulgaris* individuals and *C. lindemuthianum* strains collected in the three centers of diversity was carried out to analyze the GFG coevolution between host and pathogen. Second, a genetic dissection of anthracnose resistance specificities was performed with a recombinant inbred (RI) population derived from the cross between representative genotypes of the two major gene pools of *P. vulgaris*. Finally, degenerate primers were designed to identify RGAs co-localized with different resistance specificities underlying a potential coevolution process. We show that host-pathogen GFG coevolution has led to a differentiation of host resistance in the three centers of diversity, with each plant population containing mainly resistance against allopatric strains. A major R gene cluster, containing R genes from both Andean and Mesoamerican gene pools of *P. vulgaris*, as well as RGAs, was identified, strongly suggesting a genetic basis for the coevolution process.

RESULTS

Distribution of resistance in wild populations from the three centers of diversity of *P. vulgaris*.

A total of 26 *C. lindemuthianum* strains and 48 *P. vulgaris* wild plants coming from either Mexico, Ecuador, or Argentina

were cross-inoculated as shown in Figure. 1. A clear and consistent resistant or susceptible reaction was found for 1,137 interactions out of the 1,248 tested (Fig. 2). Resistance was observed in 70% of the interactions (Fig. 2). In order to study resistance diversity, plants were compared for their resistance reactions against all strains tested. Of 48 plants tested, 39 differed by their reaction to at least one strain (Fig. 2), indicating an important diversity for anthracnose resistance in the three centers of diversity of common bean.

Clear differences were observed depending on the origin of the plant. Indeed, the analysis of variance indicated a significant effect of host origin ($P < 0.0001$) (Table 1). This appeared to be the result of a larger number of resistant reactions of Mexican plants toward all strains tested: 81% of the interactions tested with Mexican plants were incompatible while only 68 and 63% of the interactions were incompatible with Ecuadorian and Argentinean plants, respectively. By contrast, there were no significant differences observed for pathogen origin (Table 1).

The analysis of variance also showed a highly significant ($P < 0.0001$) pathogen-by-host interaction effect (Table 1). Clear differences were observed between sympatric (within a center of diversity) and allopatric (between centers of diversity) interactions. All Mexican plants and most of the Argentinean (85%) and Ecuadorian (85%) plants were resistant to allopatric strains (Fig. 2). In contrast, over 50% of the plant reactions to sympatric strains were susceptible, showing an adaptation of the pathogen to plants of the same geographical origin. These data also indicated a differentiation between the three plant populations for resistance, each one harboring different resistance specificities against allopatric strains. Resistance specificities inefficient against most local strains were revealed after inoculation with allopatric strains.

Genomic distribution of Mesoamerican and Andean resistance specificities.

In order to study the genomic distribution of Andean and Mesoamerican resistance specificities, an RI population de-

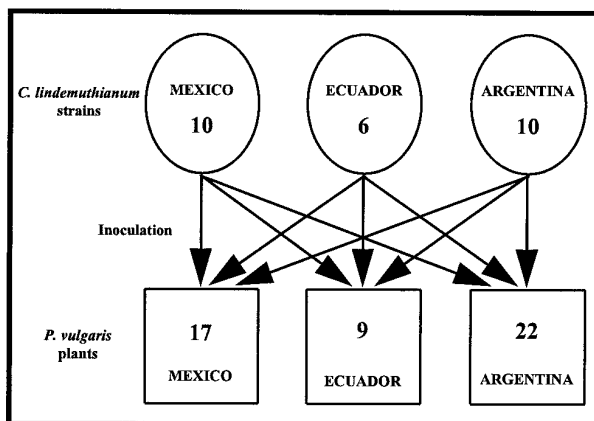


Fig. 1. Cross-inoculation experiment. *Phaseolus vulgaris* plants originating in Mexico, Ecuador, and Argentina, located in the Mesoamerican, North Andean, and South Andean centers of bean diversity, respectively, were inoculated with *Colletotrichum lindemuthianum* strains collected in the same countries. Number of strains and plants tested are indicated by circles and squares, respectively. Each strain was inoculated independently on each plant.

rived from the cross between two typical Mesoamerican and Andean cultivars, BAT93 and JaloEEP558, was used (Nodari et al. 1992, 1993). This population was previously used to construct an integrated genetic map of common bean (Freyre et al. 1998). To determine whether previously identified anthracnose R genes existed in these two genotypes, strain A7 displaying avirulence toward the *Co-2* to *Co-8* genes (Alzate-Marin et al. 1997; Kelly and Young 1996; Sicard et al. 1997; Young et al. 1998) was inoculated onto BAT93 and JaloEEP558. Strain A7 was virulent against these two genotypes, showing that both BAT93 and JaloEEP558 did not carry any of the *Co-2* to *Co-8* R genes. Similarly, strain M38 (Sicard et al. 1997), avirulent toward *Co-1*, was virulent against BAT93, indicating the absence of this specificity in BAT93. Consequently, anthracnose R genes harbored by BAT93 corresponded to new anthracnose specificities. No strains were available to determine whether JaloEEP558 contains *Co-1* (Kelly and Young 1996).

BAT93 and JaloEEP558 were inoculated with a collection of 177 strains of *C. lindemuthianum* (see Materials and Methods). Approximately half of these strains were simultaneously avirulent toward the two parents. Sixty-five (36.7%) were virulent toward JaloEEP558 but avirulent when inoculated to BAT93, 19 (10.7%) were virulent toward BAT93 but avirulent on JaloEEP558, and finally only four (2.3%) were virulent against both genotypes. These proportions, obtained on cultivated beans, confirmed the data obtained with natural populations of *P. vulgaris* and *C. lindemuthianum*. The rare cases of double

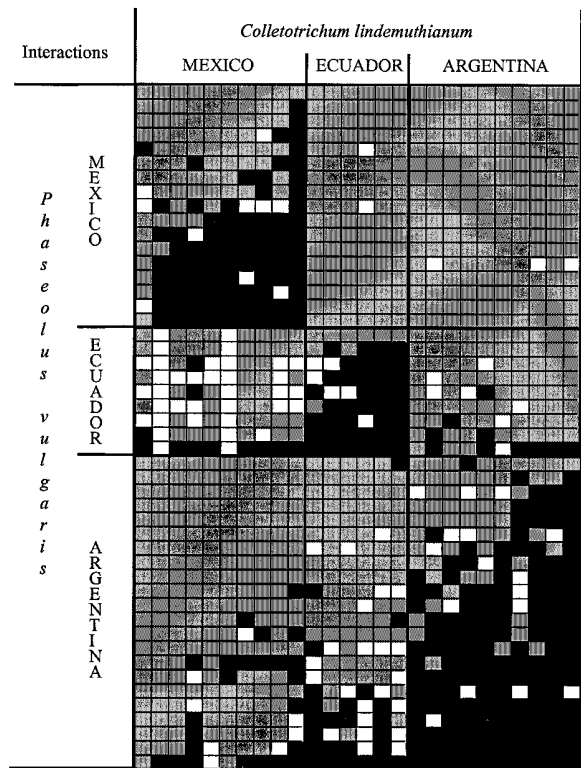


Fig. 2. Results of cross-inoculation experiment between *Phaseolus vulgaris* plants and *Colletotrichum lindemuthianum* strains collected in natural populations of *P. vulgaris* of Mexico, Ecuador, and Argentina. Each square corresponds to the interaction between one plant and one strain: A dark square = compatible; a gray square = incompatible; and a white square = an interaction that was not tested.

susceptibility reflected the fact that the pathogen is adapted either to Andean or Mesoamerican beans, but is rarely virulent on both. Moreover, a large number of the strains tested (45%) were collected in Tanzania or Europe where most *P. vulgaris* cultivars were introduced from the Andes (Gepts and Bliss 1988). Hence, it was not surprising that a larger proportion of strains were virulent toward the Andean parent, JaloEEP558.

Eight strains, four exhibiting avirulence toward the Andean parent JaloEEP558 and four against the Mesoamerican parent BAT93, were inoculated onto the RI population (Table 2). A single gene segregated for resistance to each of the eight strains. These segregating R genes were mapped with Mapmaker 3.0 onto the common bean genetic map, established in the BAT93 × JaloEEP558 RI population (Freyre et al. 1998). Resistance specificities detected in the Andean parent JaloEEP558 will be referred to as Andean specificities. In the same way, resistance specificities identified in the Mesoamerican parent BAT93 will be called Mesoamerican specificities. Two linked loci were identified at one end of linkage group B4 (Table 2; Fig. 3). One locus is characterized by the Andean specificity *Co-z*. The other locus shared Andean and Mesoamerican specificities (*Co-y* and *Co-9*). The Andean *Co-z* specificity mapped at 2.0 cM from the *Co-y/Co-9* locus on B4.

The RI population behaved identically toward each of the three strains M38, A47, and 88, suggesting a common R gene (*Co-y*). However, the population size of 77 recombinant inbred lines (RILs) was insufficient to provide unequivocal evidence for a single specificity defining resistance toward these three strains, rather than closely linked genes. The same situation occurred with strains 38, C531, E29b, and E33c (*Co-9* specificity) (Fig. 3). It was also not possible to differentiate between allelism and tight linkage for the two specificities *Co-y* (from JaloEEP558) and *Co-9* (from BAT93). Therefore, the three specificities *Co-y*, *Co-z*, and *Co-9* should be considered a minimum number of functional R genes recognized by the eight strains.

Table 1. ANOVA (analysis of variance) table for the frequency of compatible interactions between strains and plants collected in Mexico, Ecuador, and Argentina

Effects	df ^a	MS ^b	Probability
Host origin	2	2.098	<0.0001
Pathogen origin	2	0.184	0.1320
Host origin × pathogen origin	4	8.667	<0.0001

^a Degrees of freedom.

^b Mean squares.

Table 2. Disease reaction of the two parental lines BAT93 and JaloEEP558 after inoculation with eight *Colletotrichum lindemuthianum* strains

Strain	BAT93	Jalo EEP558	Name ^a	LG ^b
M38	S	R	<i>Co-y</i>	B4
A47	S	R	<i>Co-y</i>	B4
88	S	R	<i>Co-y</i>	B4
80	S	R	<i>Co-z</i>	B4
38	R	S	<i>Co-9</i>	B4
C531	R	S	<i>Co-9</i>	B4
E29b	R	S	<i>Co-9</i>	B4
E33c	R	S	<i>Co-9</i>	B4

^a Assigned names of the recognized anthracnose resistance genes.

^b Linkage group (Freyre et al. 1998).

Molecular characterization of an R gene cluster containing Mesoamerican and Andean specificities.

A molecular characterization of the R gene cluster mapped on linkage group B4 was pursued by a candidate gene approach. Two degenerate primers (see Materials and Methods) were designed based on the P-loop motif (GMGG(I/V)GKT) and the hydrophobic domain (GLPLA), which are conserved among the NBS-containing R genes *RPS2*, *RPM1*, *N*, and *L6* (Fig. 4) (Bent et al. 1994; Grant et al. 1995; Mindrinos et al. 1994; Whitham et al. 1996). The polymerase chain reaction (PCR) amplification of either BAT93 or JaloEEP558 genomic DNA with the P-loop and HD primers generated a single band of approximately 550 bp, as expected for the interval between P-loop and HD domains in the *RPS2* gene. Amplification products from each parent were cloned. Six plasmid clones, three from each parent, were randomly selected and used as probes under high stringency hybridization against blots containing either BAT93 or JaloEEP558 genomic DNA digested with *Hae*III or *Hind*III (data not shown). These six clones were grouped into three classes according to their hybridization pattern, one of them displaying the complex pattern characteristic of a multigene family. Two clones belonging to this latter class were chosen for further characterization. They were PRLJ1, cloned from JaloEEP558, the Andean genotype, and PRLB1, cloned from BAT93, the Mesoamerican genotype. PRLJ1 and PRLB1 displayed the same complex hybridization pattern, as shown for the PRLJ1 clone (Fig. 5A,B). The amino acid derived sequences of PRLJ1 and PRLB1 shared 77% identity and 91% similarity. The alignment of the derived amino acid sequences of both clones and the NBS-HD encoding region of the *RPS2* gene showed 45% sequence similarity with the strongest correspondence for the P-loop, Kinase 2a, Kinase 3a, and HD motifs (Fig. 4B). The sequences corresponding to the P-loop and HD primers were excluded from these calculations of sequence similarities, since they may not represent exact genomic sequences.

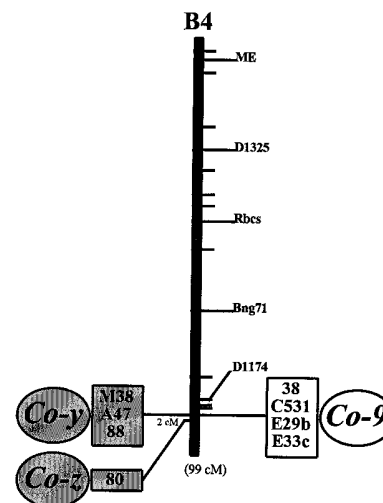


Fig. 3. Distribution of anthracnose resistance genes on the B4 linkage group. Rectangular and oval symbols represent strains and anthracnose resistance genes used in this study, respectively. Resistance specificities harbored by Andean parent JaloEEP558 are indicated on the left side of linkage groups; genes labeled on the right side originated in the Mesoamerican genotype BAT93. Distances in centimorgans (cM) are shown to the left. Size in cM of the linkage group is shown at the bottom.

The polymorphic DNA fragments revealed with the Andean clone PRLJ1 and the Mesoamerican clone PRLB1 used as probes were mapped in the BAT93 × JaloEEP558 RI population. Because identical patterns were obtained with either PRLJ1 or PRLB1, only data from hybridization with PRLJ1 are shown (Fig. 5A,B). A total of five and six polymorphic DNA fragments were obtained after hybridization on genomic DNA digested with *Hae*III and *Hind*III, respectively (Fig. 5A, B). All 11 polymorphic DNA fragments mapped on linkage group B4 within a 2.7 cM interval, which also included anthracnose resistance specificities from the Andean (*Co-y*; *Co-z*) and Mesoamerican (*Co-9*) gene pools (Fig. 5C). Three fragments from BAT93 (PRLJ1ha.d, PRLJ1hn.a, and PRLJ1hn.d) co-segregated with the Andean *Co-y* and Mesoamerican *Co-9* resistance specificities. All the other polymorphic fragments were located at a distance of 2.7 cM from this resistance locus, except band PRLJ1hn.b, which mapped at 2.0 cM from *Co-y* and *Co-9* (Fig. 5C).

DISCUSSION

Local pathogen adaptation and cost of virulence.

Cross-inoculation between 48 *P. vulgaris* plants and 26 *C. lindemuthianum* strains collected in the three centers of diver-

sity of common bean were carried out to study GFG coevolution between the two partners. To the best of our knowledge, this is the first time that cross-inoculation between a fungus and its plant host populations was assessed with such a large number of pathogen strains and over such a broad geographical scale. The cross-inoculation experiment revealed an adaptation of the pathogen to plants collected in the same country. In most cases, pathogen strains were virulent toward individual plants collected in the same country (sympatry) and avirulent toward plants collected in another country (allopatry). In this system, the pathogen appears to be ahead in its GFG coevolutionary race with its local host. This was also observed in other natural populations for the interactions between *Amphicarpea bracteata/Synchytrium decipiens* (Parker 1985) and *Linum marginale/Melampsora lini* (Burdon and Thompson 1995). An evolutionary advantage of the pathogen on its host has been suggested as a common rule in the competitive coevolution of a pathogen and its plant host (Kaltz and Shykoff 1998). This is often regarded as a consequence of the shorter generation time and the larger population size of the pathogen (Kaltz and Shykoff 1998). Furthermore, in the GFG system, the pathogen has the advantage that it needs only to lose a function (an avirulence gene) to escape host detection (Sweigard et al. 1995), while the host plant must create a new function (an R gene) to recognize a novel race of pathogen.

Adaptation of *C. lindemuthianum* to the different common bean gene pools was also observed in the two cultivated parent lines (BAT93, JaloEEP558) as well as in other cultivated lines (Pastor-Corrales et al. 1995). Other pathogens of common bean display the same adaptation. Strains of both *Phaeoisariopsis griseola*, responsible for common bean angular leaf spot, and *Uromyces appendiculatus*, the causal agent of common bean rust, were divided in two groups depending on their virulence on Mesoamerican or Andean cultivars (Guzmán et al. 1995; Steadman et al. 1995). Therefore, adaptation of pathogens to the different gene pools of cultivated common bean does not appear to depend on the dissemination rate of the fungus or on whether or not the fungus is obligate.

Interestingly, our results suggest a cost of virulence since the pathogen was rarely virulent against allopatric plants. Reports addressing the role of avirulence genes in pathogen fitness clearly demonstrated the importance of avirulence genes in endogenous biological mechanisms for *Pseudomonas syringae* pv. *vesicatoria* (Kearney and Staskawicz 1990) or *Rhynchosporium secalis* (Rohe et al. 1995). In these two studies, strains harboring avirulence genes were more competitive in compatible interactions. As proposed some years ago by Pryor (1987) and recently demonstrated in the tomato/ *Cladosporium fulvum* interaction (Lauge et al. 1998), an “avirulence” gene encodes a product that is involved in the pathogenic process, but for which the plant has developed a recognition system (an R gene). Therefore, maintaining an avirulence gene might be useful for the pathogen except when the presence of this avirulence gene leads to an incompatible interaction with the plant. Consequently, virulence might imply a cost for the pathogen.

Diversity for resistance in the three wild common bean gene pools.

A high diversity for resistance was found in the three centers of diversity. Many R genes would be required to explain the high number of differential reactions. This is in agreement

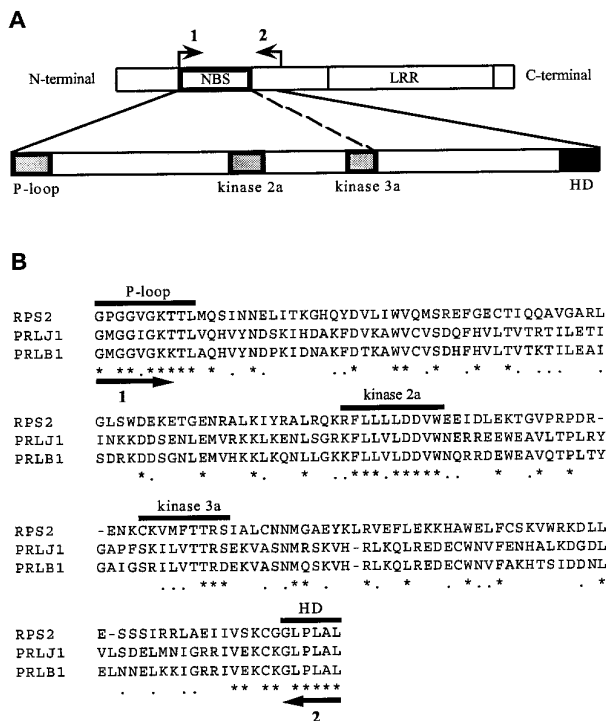


Fig. 4. A, Schematic representation of nucleotide binding site-leucine rich repeat (NBS-LRR) plant resistance genes. NBS and LRR regions are indicated by clear boxes; tripartite NBS element (P-Loop, kinase 2a, kinase 3a) is shown by shaded boxes; hydrophobic domain (HD) is indicated by a black box. Positions of the primers used for polymerase chain reaction (PCR) amplifications are indicated by arrows (1 and 2). B, Alignment of deduced amino acid RPS2-NBS gene sequence from *Arabidopsis thaliana* and of the PRLJ1 and PRLB1 clones from *Phaseolus vulgaris*. Conserved NBS and HD motifs are indicated by horizontal lines. Identical and similar amino acids are indicated by asterisks and dots, respectively. Positions of primers 1 and 2 are indicated by arrows.

with the important pathotypic diversity of the pathogen (Sicard et al. 1997) and the GFG basis of resistance in this pathosystem (Kelly and Young 1996). Our data confirm previous results in wild plant populations revealing a high level of resistance diversity between and within populations (Burdon 1997; Clarke 1997; Jana and Nevo 1991; Lenné 1988). In our study, the diversity for resistance results from variation both between and within centers of diversity.

Between centers of diversity, the difference is mainly related to the fact that plants carry R genes against allopatric pathogen strains. Historical background, migration, genetic drift, and pathogen selection pressure might be responsible for the resistance differentiation between centers of diversity. Regarding migration, our results are consistent with the prediction of a GFG meta-population model when the pathogen migrates more than its host or when host migration is low (Gandon et al. 1996). Indeed, *C. lindemuthianum* is disseminated by infected seeds and by rain at short distances (Tu 1992). Moreover, the high differentiation for neutral markers between the three centers of diversity of wild common bean suggests low gene flow at this geographical scale (Gepts 1998). Although founding effect has been regarded as a main factor explaining the difference between the three wild bean centers of diversity (Gepts and Debouck 1991; Debouck et al. 1993), it is unlikely that genetic drift alone is responsible for the resistance pattern observed. Analyses with neutral markers have shown a decreased level of diversity in the wild Andean populations (Koenig and Gepts 1989; Singh et al. 1991; Debouck et al. 1993; Velasquez and Gepts 1994). By contrast, in our study, the Andean populations appear to be as polymorphic as the Mesoamerican one for resistance diversity. Our results suggest rather that some mechanisms have favored spray of new resistance specificities in both Andean and Mesoamerican wild populations. Therefore, it is likely that, in addition to genetic drift, pathogen se-

lection pressure is responsible for the resistance pattern observed. The relative impact of these different evolutionary forces on the pattern observed is difficult to estimate. Analytical and computer simulation modeling will probably be very helpful to test the key parameters in this coevolutionary process.

Within centers of diversity, polymorphism is revealed by resistance to sympatric pathogen strains. As proposed by Burdon (1996), this high diversity for specific resistance within each center of diversity may function to reduce epidemics and help maintain natural plant populations. The presence of a few resistances efficient against sympatric strains despite the strong adaptation of the pathogen also suggested generation of new resistance specificities in the host genome. Alternatively, these resistance specificities might be ancient specificities that have not yet been overcome by the pathogen. Analysis of the cluster on linkage group B4 in the presumed ancestral populations of *P. vulgaris* (Kamir et al. 1995), as well as study of local pathogen adaptation within each country, may shed light on this alternative explanation.

Identification of a genetically and molecularly complex anthracnose resistance cluster that existed prior to the geographic separation of the two common bean gene pools.

A geographic differentiation for resistance was detected between the three centers of diversity, each plant harboring mainly resistance against allopatric strains. To understand the evolution of resistance specificities in the different gene pools, specific resistance was genetically dissected with an RI population issued from a cross between cultivars of the Andean and Mesoamerican centers of diversity. A complex locus comprising both Andean (*Co-y*; *Co-z*) and Mesoamerican (*Co-9*) resistance specificities was characterized at one end of linkage group B4. The co-localization of Andean and Mesoamerican resistance specificities strongly suggested that this cluster ex-

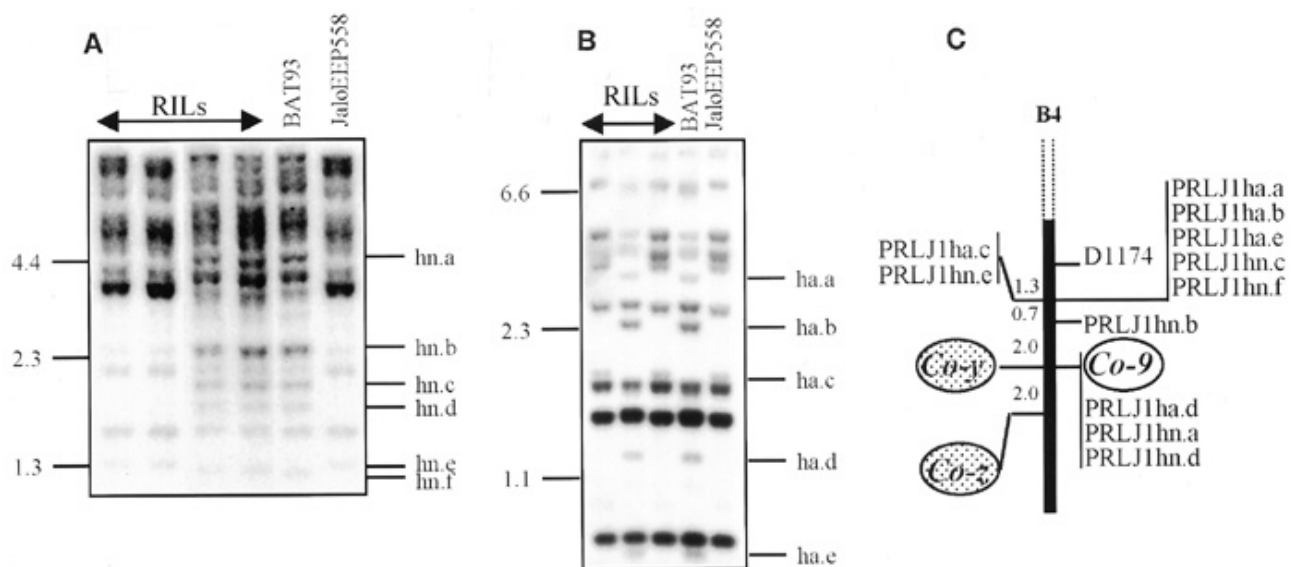


Fig. 5. **A** and **B**, Southern blot analysis of BAT93 and JaloEEP558 genomic DNA digested with either **(A)** *Hind*III or **(B)** *Hae*III and hybridized with PRLJ1 as a radiolabeled probe. Polymorphic bands PRLJ1hn.a to PRLJ1hn.f (hn.a to hn.f) and polymorphic bands PRLJ1ha.a to PRLJ1ha.e (ha.a to ha.e) are indicated on the right. **C**, Genomic location of the polymorphic bands described in **(A)** and **(B)** on linkage group B4. Polymorphic bands revealed in BAT93 (see **A** and **B**) and Mesoamerican anthracnose specificities (i.e., resistance specificities present in BAT93) are shown on right side of linkage group B4. Polymorphic bands originating in JaloEEP558, Andean anthracnose specificities (i.e., resistance specificities present in JaloEEP558), and Kosambi distances in centimorgan (cM) are shown on left side of linkage group.

isted prior to the geographic separation of the two gene pools of cultivated common bean. The evidence of independent domestication events from two wild centers of diversity (Mesoamerican and South Andean) would suggest that the origin of this cluster did also precede the separation of the wild gene pools.

The B4 cluster was independent from *Co-2*, a previously mapped anthracnose resistance locus originating from the Mesoamerican gene pool (Adam-Blondon et al. 1994). Two independent anthracnose resistance loci, *Co-3* and *Co-4*, originating from the Mesoamerican gene pool, have been shown to display two different alleles, *Co-3²* and *Co-4²*, respectively (Fouilloux 1976; Young et al. 1998), but their map locations are unknown. However, it is likely that other clusters of R genes against anthracnose exist in the bean genome. Analysis of other clusters of anthracnose R genes will allow testing the hypothesis whether the presence of both Andean and Mesoamerican specificities within the same cluster is a common rule in the bean genome.

Molecular analyses showed a high level of complexity. The B4 cluster was found to harbor 11 polymorphic DNA fragments containing NBS sequences. The use of sequence homologies from conserved motifs of cloned R genes in common bean revealed the molecular complexity of R gene loci as previously found in other plant species (Aarts et al. 1998; Botella et al. 1997; Kanazin et al. 1996; Leister et al. 1996, 1998; Ronald 1998; Shen et al. 1998; Speulman et al. 1998; Yu et al. 1996). A cluster containing RGA sequences of NBS-LRR type was identified on linkage group B4. This cluster presents both Andean and Mesoamerican resistance, suggesting that it pre-existed the separation into Andean and Mesoamerican gene pools and must have undergone part of the molecular events that led to the differentiation for anthracnose resistance between the two gene pools. The NBS sequences from the Mesoamerican and Andean parents, PRLB1 and PRLJ1, respectively, shared 91% similarity. Extensive analysis of NBS sequences from BAT93 and JaloEEP558 is actually underway to compare the polymorphism of the NBS sequences that has been selected before and after the separation of the two gene pools.

Why do plants maintain apparently unnecessary resistance specificities?

Most of the resistance specificities maintained in the different centers of diversity of common bean were efficient against allopatric strains of *C. lindemuthianum* but were overcome by local strains of the pathogen. Several reasons may account for the maintenance of these specificities that are apparently useless locally. Firstly, the observed unneeded specificities might be maintained because of their linkage to other R genes. The organization of R genes in clusters of repeated sequences as illustrated here by the cluster identified on B4 may strongly increase hitchhiking between unneeded R genes and efficient ones (against *C. lindemuthianum* or against other pathogens). Furthermore, these apparently unneeded genes might be a renewable resource for the generation of new specificities by genetic reassortment events, leading to new arrangements of preexisting functions (Borst and Greaves 1987). In fact, clustering of R genes, as observed in numerous plant species (Crute and Pink 1996; Pryor and Ellis 1993), might be a favorable factor for plants to generate new specificities at a sufficient rate to cope with an ever-changing array of pathogens. This is in agreement with comparative mapping studies of monocot RGAs, which have suggested that R genes diverge

more rapidly than the rest of the genome (Leister et al. 1998). Secondly, inefficient R genes might have other roles in the biology of the plant and might thus be involved in its fitness. LRR gene products are known to be involved in various biochemical mechanisms including signal transduction and protein/protein interactions in mammals and yeast where no correlation was found with pathogen specificity (Kobe and Deisenhofer 1994). Therefore, these genes could be simultaneously involved in different plant biochemical mechanisms including pathogen specific recognition. Thirdly, as proposed by Burdon (1996), these R genes might function locally against several pathogen strains delaying epidemics. Fourthly, although overcome, specific R genes might retain some residual resistance effects (Pedersen and Leath 1988). Finally, useless R genes might be maintained because they force the pathogen to limit the number of virulence factors it can use. Sequencing of R gene clusters, deletion of unneeded gene sequences, and fitness assays should allow a choice between these hypotheses.

Model for the evolution of a cluster of ancestral origin in two gene pools of *P. vulgaris*.

Based on the population and genetic analyses performed in this study, a putative evolutionary history of the cluster identified on B4 can be proposed. From a common origin, this cluster has evolved independently after geographical separation of bean populations leading to the three current wild gene pools. As was shown for other host-pathogen models, accumulation of single base changes (Frederick et al. 1998) combined with different mechanisms of recombination (unequal crossing-over, gene conversion) and transposition within R gene clusters (Parniske et al. 1997; Richter et al. 1995; Song et al. 1997; Sudupak et al. 1993; Thomas et al. 1997) may have led to the random generation of new sequences in each center of diversity. Among them, some were presenting new resistance specificities. Consequently, different sets of specificities were randomly generated in each center of diversity, some of these new genes being efficient against local strains of the pathogen. Therefore, selection pressures exerted by the plants were different in each center of diversity. In response, the pathogen developed new virulences, leading to the local adaptation pattern observed. Adaptation of the pathogen to the more frequent resistance haplotype created an advantage for the rare plants with new resistance specificities, as shown for another plant-pathogen interaction (Chaboudez and Burdon 1995). Therefore, new R genes spread in the plant population. Following several cycles of generation of new resistance/virulence and frequency-dependent selection, a different array of specific plant/pathogen combinations appeared and led to the observed geographic differentiation for resistance and virulence. This differentiation was probably maintained because few or no genetic exchanges for the two protagonists may have taken place between the different geographic locations.

Prospects.

The identification of a complex R gene cluster that existed prior to the geographic separation of the two common bean gene pools provides a good tool to understand the coevolution phenomenon at the molecular level. Comparison of the molecular organization of this locus in BAT93 and JaloEEP558, as well as in the presumed ancestral wild beans in Ecuador and northern Peru (Kami et al. 1995), offers prospects for un-

derstanding the evolutionary mechanisms by which this complex resistance locus has evolved in the independent bean gene pools.

MATERIALS AND METHODS

Plant and fungal material.

Plant and fungal materials were collected in natural populations of *P. vulgaris* of Mexico, Argentina, and Ecuador, located in the Mesoamerican, South Andean, and North Andean centers of diversity, respectively. The survey in Mexico was carried out in 1994 in four states: Morelos, Michoacán, Guerrero, and Jalisco. In Argentina, the collection was performed in 1992 in three regions: Tucumán, Salta, and Jujuy. Finally, in Ecuador, the material was collected in 1993 in six provinces: Pallatanga, Simbambe, Capzol, Girón, Macará, and Mulinuma. In each region, collections were made in multiple locations. Anthracniosis was found in all locations but one. The incidence of the disease ranged from one plant to all plants infected, depending on the location. In each location, seeds and infected material were harvested separately from each plant. Strains of *C. lindemuthianum* were obtained from infected pods as described in Sicard et al. (1997). A total of 48 plants and 26 strains were chosen to test for resistance (Fig. 1). The samples were selected to represent different geographical locations with no regard to the incidence of the disease or the severity of the symptoms. Out of the 1,248 interactions tested, only 33 interactions were between a plant and a strain collected from the same location.

The breeding line BAT93 and the landrace JaloEEP558 were provided by S. Singh (CIAT, Cali, Colombia). Based on phenotypic, biochemical, and molecular data, BAT93 presents all the characteristics of a Mesoamerican type and JaloEEP558 of an Andean type (Nodari et al. 1993). Seventy-seven F_2 recombinant inbred lines (RILs), developed at the University of California, Davis, and derived from a cross between BAT93 and JaloEEP558, were used to map resistance specificities and RGA sequences on the bean integrated linkage map (Freyre et al. 1998). The 177 *C. lindemuthianum* strains used to search for differential specificities between the two parental genotypes BAT93 and JaloEEP558 were obtained from the Orsay fungal library. They include strains from Europe, Latin America, and Southeast Africa, isolated from either wild or cultivated beans (Fabre et al. 1995; Sicard et al. 1997).

Pathogenicity tests.

For wild bean seeds, homogeneous germination was obtained by immersing the seeds in liquid nitrogen for 10 s. Five seeds of each plant were sown in moist vermiculite and plants were grown at 23°C, with 16 h light under fluorescent tubes ($166 \mu\text{E s}^{-1} \text{m}^{-2}$). After 7 days, seedlings were sprayed with a 10^6 conidia per ml suspension of *C. lindemuthianum* and incubated at 19°C, 90% controlled humidity, under the same light conditions as described for the germination of seedlings. Symptoms were scored 7 days after inoculation. Plants without visible symptoms or showing limited necrotic lesions were scored as resistant. Plants with large, sporulating lesions and dead plants were scored as susceptible. Therefore, plant reaction was scored qualitatively (infected or not) but not quantitatively. When the five seedlings showed heterogeneous phenotypes, the plant reaction was not recorded. The same procedure

was used for pathogenicity tests on BAT93, JaloEEP558, and the derived RIL population except that seeds were not immersed in liquid nitrogen. For R gene nomenclature (Alzate-Marin et al. 1997; Kelly and Young 1996; Young et al. 1998), new specificities were labeled with numbers (for example *Co-9*) while putative new specificities were labeled with letters (for example *Co-x*).

Molecular analyses.

Degenerate oligonucleotide primers P loop-Sense (5-GGNATGGG(C/T)GG(G/T/C)(A/G)T(A/T/C)GG(C/T)AA(A/G)AC-3) and HD-Antisense (5-CA(A/G)(A/C)GC(C/T)AA(A/T)GG(C/T)AA(G/A/T)CC-3), were designed from the P-loop NBS (GMGG(I/V)GKT) and the hydrophobic domain (GLPLA), respectively, which are conserved among the NBS-containing R genes *RPS2*, *RPM1*, *N*, and *L6* (Bent et al. 1994; Grant et al. 1995; Mindrinos et al. 1994; Whitham et al. 1996). PCRs were processed in a 50- μl total reaction mixture containing 40 ng of total DNA extracted from the bean genotypes BAT93 or JaloEEP558, $1\times$ PCR buffer (Eurogentec, Seraing, Belgium), 50 pmol of each primer, 1.5 mM MgCl_2 , 0.1 mM concentrations of each dNTP, and 0.2 U of *Taq* DNA polymerase (Eurogentec). Amplification reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT) programmed for 35 cycles as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Amplification products were directly cloned into the pAT-ag vector from the LigATor cloning kit (R&D Systems Europe Ltd., Abingdon, UK). Double-stranded sequencing of plasmid DNA was performed with the ABI PRISM Dye Terminator kit (Perkin-Elmer) in a Perkin-Elmer/Cetus 9600 thermal cycler, and analyzed in the Applied Biosystems (Roissy, France) 373 automated sequencer.

DNA extraction, Southern blot, and hybridization experiments were carried out as already described (Geffroy et al. 1998).

Data analysis.

Pathogenicity tests involving natural populations were analyzed with a two-way analysis of variance (ANOVA) model (origin of the plant and origin of the fungal strain) with an interaction component between the origin of the plant and the origin of the fungal strain (the GLM procedure of Statistical Analysis System [SAS], 1989 SAS/STAT User's Guide, version 6, 4th ed., SAS Institute, Cary, NC). The three effects tested (origin of plants, fungal strains, and interaction between plant and fungal origin) were considered as fixed effects. The distribution of the dependent variable (frequency of susceptible reaction to one strain in each center of diversity) was tested for normality by the method of Shapiro and Wilk (1965). The analysis was made after $\arcsin\sqrt{}$ transformation of the dependent variable to meet the assumptions of the parametric tests. To map resistance specificities and RGA sequences on the integrated bean genetic map, MAPMAKER software version 3.0 was used (Lander et al. 1987). Distances between markers were presented in Kosambi centimorgans (Kosambi 1944). Sequence homology analysis was carried out in GenBank, SwissProt, and EMBL data bases, with the BLAST algorithm (Altschul et al. 1990). Sequence alignment of deduced amino acid sequences was obtained with the CLUSTAL software (Higgins and Sharp 1988).

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