Theor Appl Genet (1998) 96: 494–502

V. Geffroy · F. Creusot · J. Falquet · M. Sévignac
A-F. Adam-Blondon · H. Bannerot · P. Gepts · M. Dron

A family of LRR sequences in the vicinity of the *Co-2* locus for anthracnose resistance in *Phaseolus vulgaris* and its potential use in marker-assisted selection

Received: 26 June 1997 / Accepted: 13 October 1997

Abstract Molecular markers offer new opportunities for breeding for disease resistance. Resistance gene pyramiding in a single cultivar, as a strategy for durable resistance, can be facilitated by marker-assisted selection (MAS). A RAPD marker, ROH20^{450}, linked to the Mesoamerican *Co-2* anthracnose resistance gene, was previously transformed into a SCAR marker, SCH20. In the present paper we have further characterized the relevance of the SCH20 SCAR marker in different genetic backgrounds. Since this SCAR marker was found to be useful mainly in the Andean gene pool, we identified a new PCR-based marker (SCAreoli) for indirect scoring of the presence of the *Co-2* gene. The SCAreoli SCAR marker is polymorphic in the Mesoamerican as well as in the Andean gene pool and should be useful in MAS. We also report that *PvH20*, the cloned sequence corresponding to the 450-bp RAPD marker ROH20^{450}, contains six imperfect leucine-rich repeats, and reveals a family of related sequences in the vicinity of the *Co-2* locus. These results are discussed in the context of the recent cloning of some plant resistance genes.

Communicated by M. A. Saghai Maroof

V. Geffroy (✉)[1] · F. Creusot · J. Falquet · M. Sévignac
A.-F. Adam-Blondon[2] · M. Dron
LPPM, IBP, Bât, 630, Université Paris Sud, 91405 ORSAY Cedex, France
E-mail: geffroy@lovelace.infobiogen.fr

H. Bannerot
Station d'Amélioration des Plantes, INRA, Route de Saint-Cyr, 78 026 Versailles, France

P. Gepts
Department of Agronomy and Range Science, University of California, Davis, CA 95616-8515, USA

Present addresses:
[1] Station de Génétique et Amélioration des Plantes, INRA, Domaine de Brunehaut, Estrées-Mons, 80 200 Peronne, France
[2] Laboratoire de Recherches Viticoles, INRA-ENSAM, 2 Place Viala, 34 060 Montpellier Cedex1, France

Key words Disease resistance · Anthracnose (*Colletotrichum lindemuthianum*) · Common bean (*Phaseolus vulgaris*) · Marker-assisted selection · Leucine-rich repeat motif

Introduction

Common bean (*Phaseolus vulgaris*) is a major source of protein for human consumption in many parts of the world (FAO 1980). Anthracnose, caused by the specialized hemibiotrophic fungus *Colletotrichum lindemuthianum*, is the most important disease of beans throughout the world, especially in tropical areas of Latin America and Eastern Africa where the bean is one of the major staple crops (Pastor-Corrales and Tu 1989). Because chemical control is expensive and the generation of pathogen-free seeds is often difficult in developing countries, genetic resistance represents the most reliable control strategy (Pastor-Corrales and Tu 1989). Use of resistant genotypes is not only a cost-effective but also a biologically safe management strategy.

The genetics of anthracnose resistance has been studied for a long time (McRostie 1919). This host-pathogen interaction was the first report of race-cultivar specificity (Barrus 1911). Several dominant genes which condition resistance against different races of the fungus have been described (McRostie 1919; Mastenbroek 1960; Bannerot 1965; Bannerot et al. 1971; Fouilloux 1976, 1979; Pastor-Corrales et al. 1994; Young and Kelly 1996 a), strongly suggesting a gene-for-gene-type resistance (Flor 1955) for this interaction. In Europe (Fouilloux 1979), anthracnose has been controlled for 30 years by a single dominant gene called *Are* (Mastenbroek 1960) and recently re-named *Co-2* (Kelly and Young 1996). In North America, breeding for anthracnose resistance has focused on the use of the *Co-1*

(McRostie 1919) and *Co-2* genes, but recently Kelly et al. (1994) have reported the occurrence of races that overcome either *Co-1* or *Co-2*. One response to the short-term protection conferred by single genes, is to pyramid specific resistance genes (Nelson 1978; Mundt 1990). However, incorporating more than one resistance gene into a single breeding line is a difficult, time- and space-consuming task because the epistatic interactions between resistance genes require testcrossing and progeny testing with different strains of the pathogen.

Molecular approaches offer new opportunities for the manipulation (by marker-aided selection or MAS) and the understanding of resistance genes (Melchinger 1990; Michelmore 1995). Resistance genes involved in gene-for-gene interactions have intriguing common features as shown by numerous genetic and molecular studies. They are often localized at complex loci, displaying either a multiallelic structure or clustering with each allele or gene leading to a different specificity (Pryor and Ellis 1993). Some genes could therefore be related in function and evolution (Pryor 1987). Recent cloning of such genes has confirmed this hypothesis. Two main families have been described. The *Pto* gene from tomato contains a serine/threonine protein kinase domain (Martin et al. 1993). Resistance genes against very different pathogens (bacteria, fungi, virus and nematode) isolated from *Arabidopsis* (Bent et al. 1994; Mindrinos et al. 1994; Grant et al. 1995; Parker et al. 1997), flax (Lawrence et al. 1995), tobacco (Whitham et al. 1994), tomato (Jones et al. 1994; Ori et al. 1997) and sugar beet (Cai et al. 1997) share a leucine-rich repeat region (LRR). The shared LRR motif suggests a possible common mechanism for the function of these resistance genes. LRR motifs are found in many plant and animal proteins and are believed to mediate protein-protein interactions (Kobe and Deisenhofer 1994).

The *Co-2* gene has been genetically mapped at one end of the linkage group P1 of the Paris bean map (Adam-Blondon et al. 1994 b), and at one end of linkage group D1 of the Davis bean map (Nodari et al. 1993; Freyre et al. 1996). Three RAPD (random amplified polymorphic DNA) markers were found closely linked to the *Co-2* gene (Adam-Blondon et al. 1994 a; Young and Kelly 1996 b). One of them, $ROH20^{450}$, was transformed into a SCAR (sequence characterized amplified region) marker, referred to as SCH20 (Adam-Blondon et al. 1994 a).

Two major gene pools, known as the Andean (South America) and Mesoamerican (Mexico and Central America) gene pools, have been identified for *P. vulgaris* (Singh et al. 1991). Because of this, molecular markers linked to resistance genes may sometimes be of limited value in MAS since they may be present in susceptible genotypes belonging to the gene pool from which the resistance genes of interest were derived. For example, the RAPD marker linked to the Andean rust resistance gene *Ur-4* (formerly *Up-2*) is monomorphic in an Andean background (Miklas et al. 1993). Thus, this RAPD marker can only be used for the introgression of *Ur*-4 into Mesoamerican beans.

In the present paper, we characterize the usefulness of the SCH20 SCAR marker in different genetic backgrounds. Since this marker was found to be useful mainly in the Andean gene pool, we have developed a new PCR-based marker for indirect scoring of the presence of the *Co-2* gene, which is useful in the Mesoamerican as well as in the Andean gene pool. We also report that *PvH20*, the cloned sequence corresponding to the RAPD marker $ROH20^{450}$, contains six Leucine-rich repeats, and reveals a family of related sequences in the vicinity of the *Co-2* locus, consistent with a *PvH20* family member residing at the *Co-2* locus.

Materials and methods

Bean lines

A range of French common bean cultivars were used in this study (the seed company is indicated in parentheses): Aiguille Vert (Boret, Beaufort-en-Vallée, France); Adagio, Masaï, Talisman (Caillard, Les Ponts-de-Cé, France); Ardinal, Argus, Corel, Fruidor, Janus (Clause, Brétigny-sur-Orge, France); Calypso, Coktel, Filao, Vilbel (Vilmorin, La Ménitrée, France); Cupidon (Gautier, Eyragues, France); and La Victoire (Tezier, Valence-sur-Rhône, France). Genotypes derived from INRA breeding programs, such as AFN, EO2, My and TO, were also included (Fouilloux 1979). Cornell49242 is a line from Venezuela in which the *Co-2* gene was first identified (Mastenbroek 1960). G 2333 is a germplasm accession of Mexican origin which is resistant to a broad range of *C. lindemuthianum* strains (Pastor-Corrales et al. 1994). The breeding line BAT93 and the landrace JaloEEP558 were provided by S. Singh (CIAT, Cali, Colombia). Based on phaseolin electrophoretic profiles and genealogical data, Adagio, AFN, Aiguille vert, Ardinal, Argus, Calypso, Coktel, Corel, Cupidon, Filao, Fruidor, JaloEEP558, Janus, La Victoire, Masaï, Talisman, and Vilbel belong to the Andean gene pool, whereas BAT93, Cornell49242, EO2, G 2333, My, TO, have a Mesoamerican origin.

Near-isogenic lines (NILs)

Three pairs of near-isogenic lines (NILs) differing at the *Co-2* locus were bred by H. Bannerot (INRA, Versailles): P12R and P12S, Co2LVE, Co2LVA and La Victoire. Co2LVE and Co2LVA result from the introgression, via backcrossing, of the single major dominant gene *Co-2*, derived from the donor EO2 (for Co2LVE) and AFN (for Co2LVA), in the Andean cultivar La Victoire, which carries no known anthracnose resistance genes. Co2LVE is a BC8-F5 and Co2LVA is a BC6-F4. P12R results from the introgression of the *Co-2* resistance gene from Cornell49242 in the Andean "Processor" background, and is a BC12-F15.

Segregating populations

A backcross population of 72 individuals originating from a cross between EO2 (*Co-2*, resistant) and Corel (*co-2,* susceptible) (Adam-Blondon et al. 1994 b), was used to check the linkage between

markers and the *Co-2* resistance gene. Seventy seven recombinant inbred lines (RILs), developed at the University of California, Davis, USA, (Nodari et al. 1993) and derived from a cross between BAT93 and JaloEEP558, were used to localize markers on the bean integrated linkage map (Freyre et al. 1996). Neither BAT93 nor JaloEEP558 express the *Co-2* resistance gene.

DNA extraction

Nuclear DNA was isolated as follows. Two grams of leaves were ground in liquid nitrogen. The powdered tissue was transferred into 50 ml of cold buffer 1 (10 mM Tris pH 7.5; 15 mM KCl; 15 mM NaCl; 0.15 mM spermine; 0.5 mM spermidine; 2 mM EDTA; 0.5% triton-X100 and 4.5% glucose) and filtered on blutex. The filtrate was centrifuged for 10 min at 2600 rpm and 4°C. The supernatant was discarded. The pellet was rinsed with 5 ml of buffer-1 three times and was finally re-suspended into 1 ml of distilled water. Afterwards, a CTAB extraction procedure was performed as described in Doyle and Doyle (1987) except that the chloroform-isoamyl alcohol extraction step was replaced by a chloroform-octanol (24 : 1) extraction step.

PCR amplification

ROH20^{450} stands for the RAPD marker obtained by PCR amplification using the Operon 10-mer oligonucleotide H20 as a primer (Operon Technologies Inc., Alameda, USA), as indicated in Adam-Blondon et al. (1994 a). *PvH20* corresponds to the ROH20^{450} DNA fragment inserted into the pBluescript KSII vector (Stratagene Cloning system, La Jolla, Calif. USA); "*Pv*" stands for *P. vulgaris*. SCH20-1 (5′GGGAGACATCCATCAGACAACTCC3′), and SCH20-2 (5′GGGAGACATCTTCATTTGATATGC3′) are two 24-mer primers deduced from the ROH20^{450} sequence (Adam-Blondon et al. 1994 a). Areoli-1 (5′GTATCCATTTGAAGGAGCT3′), is a 19-mer primer derived from the analysis of the Lambda genomic clones containing the ROH20^{450} sequence (Creusot et al. 1997). These three primers were supplied by Eurogentec (Seraing, Belgium). SCH20 is the SCAR marker derived from *Dde*I digestion of the amplification product obtained using SCH20-1 and SCH20-2 as primers. The PCR conditions were as described in Adam-Blondon et al. (1994 a), except that PCR reactions were carried out using a GeneAmp PCR System 9600 (Perkin Elmer Corporation, Norwalk, USA). SCAreoli is the SCAR marker obtained after *Dra*I digestion of the PCR product synthesized using the Areoli-1 and SCH20-1 primers. This SCAR analysis was as described in Paran and Michelmore (1993) except that the annealing temperature of the PCR reaction was 58°C.

Southern analysis

Approximately 2 µg of genomic bean DNA was digested by restriction enzymes according to the manufacturer (GIBCO BRL, Gaithersburg, Maryland). DNA electrophoresis and blot steps were performed according to Reed and Man (1985). The *PvH20* sequence was used as a probe in the hybridization experiments. An aliquot of 25–30 ng of the purified fragment was labelled using the Ready-to-Go labelling kit (Pharmacia, Uppsala, Sweden). Pre-hybridization and hybridization steps were performed at 65°C in a solution containing 5 × SSC, 5% SDS, and 5 × Denhardt's in a hybridization oven (Appligene, Illkirch, France). Three 20-min washes, at low stringency, were performed in 2 × SSC and 0.5% SDS at 65°C. When high-stringency washes were needed, a solution of 0.5 × SSC and 0.5% SDS was used at 65°C. X-ray films (X OMAT-AR Kodak films, Rochester, N.Y.) were then exposed to the filters at − 80°C using an intensifying screen.

Sequence analysis

The plasmid containing the *PvH20* fragment was purified by using the Wizard kit (Promega, Paris, France). Double-stranded sequencing was carried out by the dideoxy chain-terminator method using the Dye Primer Cycle Sequencing Kit (Perkin-Elmer Corporation, Norwalk, USA). The DNA sequence was analyzed on a 373A DNA sequencer (Applied Biosystems, Roissy, France). Sequence alignment was carried out using the Bestfit program of the Genetics Computer Group (Madison, WIS.) package (Devereux et al. 1984).

Linkage analysis

Marker order and genetic distances were estimated using MAPMAKER software version 3.0 (Lander et al. 1987). Distances between markers were presented in Kosambi centimorgans (cM) (Kosambi 1944).

Results and discussion

PvH20 marker sequences

PvH20 is a 450-bp fragment corresponding to the RAPD marker ROH20^{450} (Adam-Blondon et al. 1994 a). Only one recombinant out of 120 individuals was found between ROH20^{450} and the *Co-2* resistance locus. The nucleotide sequence of *PvH20* revealed an open reading frame encoding a putative polypeptide of 148 amino acids (Fig. 1 A). The predicted polypeptide sequence contained six imperfect leucine-rich repeats (LRR) (Fig. 1 B). Interestingly, most of the resistance genes characterized to-date encode proteins that contain a leucine-rich repeat domain (Bent et al. 1994; Jones et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994; Grant et al. 1995; Lawrence et al. 1995; Dixon et al. 1996; Cai et al. 1997; Ori et al. 1997; Parket et al. 1997). Pairwise alignment of the *PvH20* amino-acid sequence with the LRR domain of cloned resistance gene products gives an overall similarity of 41.9–47.9%. More significantly, the spacing of leucine and cysteine residues, as shown in bold letters in Fig. 1 B, is typical of the structural arrangement of previously described LRRs, including those found in resistance genes.

PvH20-related sequences in the common bean genome

High-stringency hybridization experiments with *PvH20* as a probe were performed with restricted genomic DNA of various *Co-2* or *co-2* bean genotypes. After hybridization, three patterns were observed (Fig. 2). All genotypes with the *Co*-2 allele presented a 1.7-kb *Dra*I band (Fig. 2). Susceptible *co-2* individuals presented either a 7-kb *Dra*I band or a null allele (Fig. 2). This was confirmed by similar hybridization experiments with *Dra*I-restricted genomic DNA from 11 French bean cultivars with or without a *Co*-2 introgression (data not shown). The ten cultivars carrying *Co-2*

Fig. 1A, B Nucleotide sequence and deduced amino-acid sequence of *PvH20*. The amino-acid residues are shown in single-letter code. The amino-acid sequence was obtained using the TRANSLATE program of the Genetic Computer Group Software package. The *arrows* indicate the position of the SCH20-1 and SCH20-2 oligonucleotides used as primers in the SCAR analysis. **B** LRR organization of the protein sequence. The conserved amino acids characteristic of the LRR structure are highlighted in *bold letters*. *1 to 6* are the numbers of LRR

A

```
    E   T   S   S   F   D   M   L   L   S   H   P   L   H   S   L   G   I
GG GAG ACA TCT TCA TTT GAT ATG CTC CTA TCT CAT CCT CTT CAT TCC TTA GGT ATT
                   SCH20-2

Y   C   C   P   G   M   N   I   P   M   N   H   G   Y   P   F   L   V   E
TAT TGT TGT CCG GGC ATG AAT ATT CCC ATG AAC CAT GGT TAC CCT TTC CTT GTA GAA

L   R   I   I   Q   C   C   D   S   L   T   N   F   P   L   D   L   F   P
TTG CGT ATC ATT CAA TGT TGT GAC TCT CTC ACT AAC TTC CCT CTA GAC TTG TTC CCA

K   L   C   S   L   R   L   D   G   C   Y   N   L   Q   M   I   T   Q   G
AAA CTC TGT TCC CTT AGA TTA GAC GGA TGT TAT AAC CTA CAG ATG ATA ACA CAG GGG

H   P   H   G   H   L   K   S   L   S   I   R   K   C   S   E   L   E   L
CAC CCT CAC GGT CAT TTG AAG AGT CTG AGT ATT CGA AAA TGC TCT GAA TTG GAA TTA

F   P   D   E   G   L   F   A   P   Q   L   E   T   F   S   I   E   G   L
TTT CCC GAT GAA GGA TTA TTT GCA CCT CAG CTA GAG ACA TTT TCG ATT GAA GGA TTA

K   K   W   K   S   M   P   K   R   M   Y   A   L   F   P   S   L   N   R
AAG AAA TGG AAA TCA ATG CCT AAA CGC ATG TAC GCC CTC TTT CCA TCT CTG AAT CGT

L   S   I   K   D   C   P   G   V   E   L   S   D   G   C   L
CTG TCT ATA AAG GAT TGT CCA GGT GTG GAG TTG TCT GAT GGA TGT CTC CC
                                  SCH20-1
```

B

```
1    ETSSFDMLLSHPLHSLGIYCCPGMNIP
2         MNHGYPFLVELRIIQCCDS-LTNFPLDLFP
3              KLCSLRLDGCYN--LQMITQGHP
4            HGHLKSLSIRKCSE--LELFP
5          DEGLFAPQLETFSIEGLKKWKSMPKRMYALFP
6              SLNRLSIKDCPGVELSDGCL
```

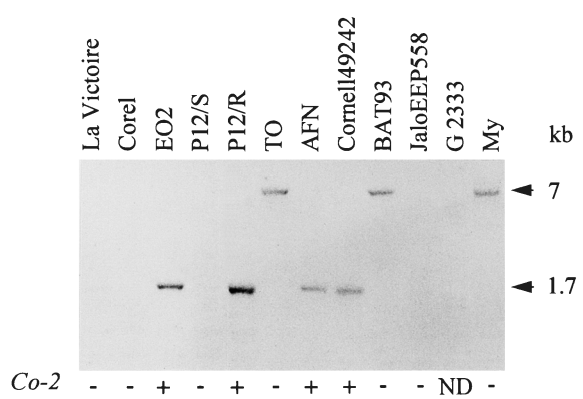

Fig. 2 Autoradiogram of high-stringency hybridization of *PvH20* to *Dra*I-digested DNA from 12 bean lines. Four of these lines possess the *Co-2* gene (+) while seven of them have the susceptibility allele *co-2* (−). *PvH20* failed to hybridize or hybridized to a 7-kb fragment or to a 1.7-kb fragment. *ND*: not defined, resistance genes have not been characterized in G 2333. La Victoire, Corel, P12/S, P12/R, AFN, and JaloEEP558 are Andean genotypes, while EO2, TO, Cornell49242, G 2333, and My are Mesoamerican genotypes

(Ardinal, Janus, Talisman, Coktel, Vilbel, Filao, Calypso, Argus, Cupidon, and Fruidor) showed the 1.7-kb band while the susceptible Andean cultivar Masaï presented the null allele. *PvH20* revealed a polymorphism between BAT93 and JaloEEP558, which are both *co-2* susceptible (Fig. 2). This 7-kb band present in BAT93 (referred to as PvH20ST in Fig. 3) was mapped near the end of linkage group B1 on the RIL population derived from a cross between the two genotypes. It co-segregated with the SCH20 SCAR marker and also with the 450-bp RAPD marker ROH20, present in BAT93 (Fig. 3 A). Susceptible *co-2* genotypes of Mesoamerican origin presented the 7-kb band or the null allele whereas susceptible genotypes of Andean origin presented only the null allele. All *Co-2* resistant genotypes tested harboured the 1.7-kb *Dra*I band. This reinforces the conclusion that *PvH20* is tightly linked to the *Co-2* gene. To determine whether sequences related to *PvH20* exist elsewhere in the common-bean genome, low-stringency hybridizations were performed with genomic DNA isolated from *Co-2* or *co-2* bean genotypes (Fig. 4).

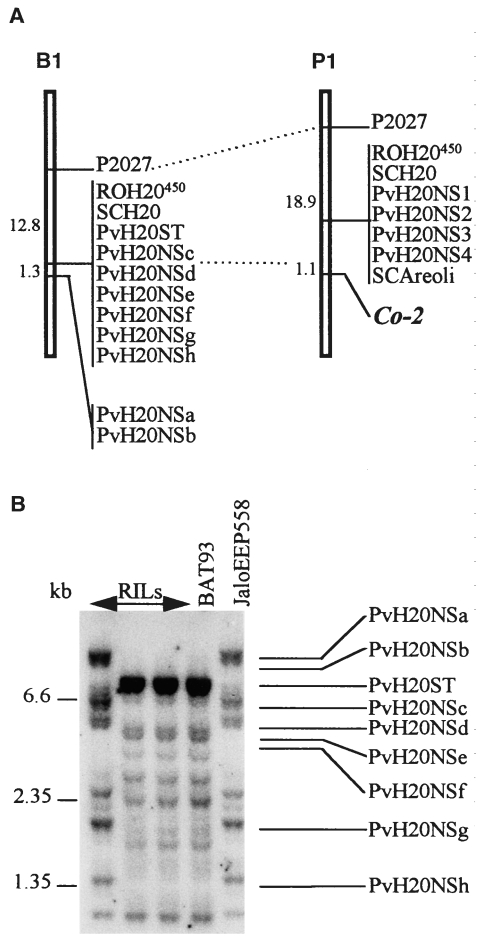

Fig. 3A, B Partial map of the end of linkage group 1 based on two different segregating populations. *B1* and *P1* were established respectively with the BAT93 × JaloEEP558 RIL population (Freyre et al. 1996) and the EO2 × Corel backcross population (Adam-Blondon et al. 1994 b). The distance between the markers is shown at the left of the linkage group in Kosambi cM. Probe P2027 establishes a correspondence between linkage groups B1 and P1. ROH20^{450} represents the RAPD marker (450-bp amplified fragment) present in EO2 and BAT93. SCH20 represents the SCAR marker generated with the SCH20-1 and SCH20-2 primers after *Dde*I digestion. For PvH20NS a to h and PvH20ST see Fig. 3 B. PvH20NS1 to 4 correspond to bands scored under low-stringency hybridization conditions with *Hae*III-digested DNA from EO2 × Corel lines. SCAreoli is the PCR marker generated with the Areoli-1 and SCH20-1 primers after *Dra*I restriction. **B** Hybridization patterns of *Dra*I-restricted DNA from BAT93, JaloEEP558 and three corresponding RILs with probe *PvH20* under low-stringency conditions. PvH20NS a to h and PvH20ST represent bands scored under low-stringency hybridization conditions, PvH20ST is the 7-kb *Dra*I band present in BAT93 after high-stringency hybridization conditions. Molecular weights in kilobases are indicated to the left

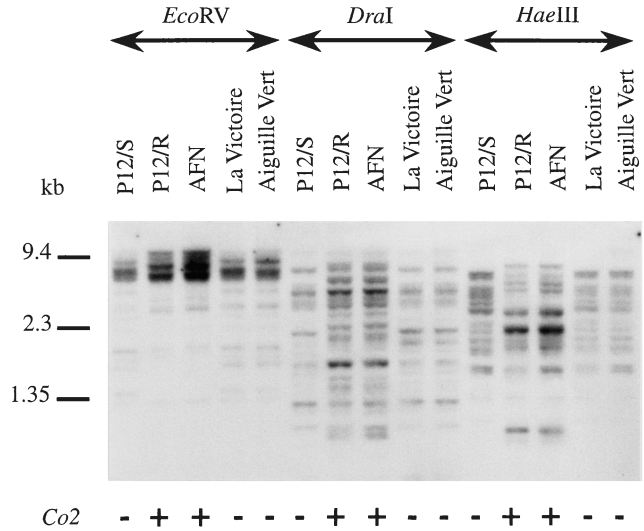

Fig. 4 Low-stringency hybridization of *PvH20* to restricted DNA from various Andean bean lines in which the Mesoamerican *Co-2* gene has been introgressed (+) or not (−). *P12/S* and *P12/*R are NILs diverging for the *Co-2* gene. Molecular weights in kilobases are indicated to the left

At low stringency, *PvH20* revealed a complex pattern of about 20 *Dra*I bands ranging from 0.7 to 9.1 kb, either in resistant (P12/R, AFN) or in susceptible genotypes (P12/S, La Victoire, Aiguille Vert) (Fig. 4). This suggested that the *PvH20* sequence exists as a family of repeated elements in the bean genome. All resistant genotypes known to harbour the *Co-2* gene presented an identical hybridization pattern which was clearly different from the susceptible genotypes (Fig. 4). This was obvious when the three pairs of NILs, P12/R and P12/S (Fig. 4), Co2LVA and La Victoire, Co2LVE and La Victoire, were tested (data not shown). Since NILs are in theory genetically identical except in the introgressed region, the latter data suggested the occurrence of a family of *PvH20*-related sequences in the vicinity of the *Co-2* locus. However, among *co-2* genotypes, different patterns were obtained according to the gene pool. Andean *co-2* genotypes presented nearly identical hybridization patterns (Fig. 4), while polymorphic patterns were revealed among *co-2* Mesoamerican genotypes (different from the *Co-2* pattern) (data not shown). It is therefore in the Mesoamerican gene pool, from which *Co-2* originates, that *PvH20* exhibited a high level of polymorphism.

To confirm the clustering of *PvH20*-related sequences in the vicinity of the *Co-2* locus, the polymorphic bands observed after low-stringency hybridization were mapped in two segregating populations. The backcross progeny (EO2 × Corel) segregating for the *Co-2* gene was used to check for such linkage. Four *Hae*III polymorphic bands (PvH20NS 1–4) were scored and co-segregated with the SCH20 SCAR marker on linkage group P1 (Fig. 3 A). The family of *PvH20*-related sequences thus appears to be clustered in the vicinity of the *Co-2* locus. The RIL progeny derived from a cross between the Mesoamerican breeding line BAT93 and the Andean landrace JaloEEP558 were also analyzed to locate this family on the integrated common bean linkage map (Freyre et al. 1996). Neither parent expresses the *Co-2* allele. Nine *Dra*I

polymorphic bands (PvH20NSa to h, and PvH20ST) differentiated these two genotypes (Fig. 3 B). They all mapped to one end of linkage group B1 where the *Co-2* locus has been located (Fig. 3 A). Seven of the bands co-segregated with the SCH20 SCAR marker (after *Dde*I digestion) (Fig. 3 A). The two remaining bands (PvH20NSa and PvH20NSb) were located 1.3 cM from the main cluster (Fig. 3 A).

Polymorphism between Andean and Mesoamerican bean lines

Co-2 has a Mesoamerican origin (Mastenbroek 1960), but has been introgressed by breeders into many cultivars of Andean origin. The *PvH20* sequence may have evolved differently in the two major gene pools of *P. vulgaris*. It appears to be absent from the Andean gene pool under high-stringency conditions. In the Mesoamerican gene pool, it might have changed through point mutations (explaining the difference between the 1.7-kb and the 7-kb alleles) or through major changes such as unequal crossing-over or deletions leading, for instance, to the null allele (G 2333). The *PvH20* sequence thus seems to be more polymorphic in the Mesoamerican gene pool than in the Andean gene pool, under high-as well as low-stringency hybridization conditions. However, this interpretation must be viewed with caution for two reasons. Firstly, French bean cultivars analyzed in this study represent only a small sample of Andean diversity. Secondly, these cultivars have undergone successive selection programs involving introgressions from the Mesoamerican gene pool. Further characterization of the *PvH20* sequences is underway in our laboratory on a set of wild bean accessions collected in the regions of origin of *P. vulgaris* (from Mexico to Argentina).

Existence of multigene loci for other disease resistance genes and potential role in evolution

The *PvH20* sequence hybridizes to a linked multigene family in the vicinity of the *Co-2* locus, contains six leucine-rich repeats, and shares some degree of overall amino-acid sequence similarity with the LRR domains of the resistance genes cloned from different plant species. These observations, together with the fact that resistance genes (to one or more unrelated pathogens), are frequently clustered (Dickinson et al. 1993; Pryor and Ellis 1993), suggest that the *PvH20*-related sequences may be candidate resistance genes. Such candidate resistance genes have also been reported in soybean (Kanazin et al. 1996; Yu et al. 1996). Similar multi-band patterns have been observed for the hybridization of restricted genomic DNA using resistance genes such as *Pto* (Martin et al. 1993), *N* (Whitham et al. 1994), *Cf-9* (Jones et al. 1994), *L6* (Lawrence et al. 1995), and *Cf-2* (Dixon et al. 1996) as probes. The function of these related sequences is not yet known except for the *Fen* gene, one of the *Pto* homologs (Martin et al. 1994), and for the *L6*-related sequences (Ellis et al. 1995). Similarly, in maize, at the top of chromosome 10, several rust resistance genes seem to be arranged in a tandem array at the *Rp1* locus (Sudupak et al. 1993). The sequence of a marker tightly linked to *Rp1* was similar to that of a serine-threonine protein kinase of the *SNF1* family (Bennetzen et al. 1994). At least 12 homologous sequences to this kinase are present in the maize genome, most of them mapping close to the *Rp1* locus (Bennetzen et al. 1994). Since serine-threonine protein kinases represent another class of resistance genes (Martin et al. 1993), this *SNF1* family could also encode for resistance genes (Bennetzen et al. 1994). Tandem repeats could increase the rate of evolution through unequal crossing over (Sudupak et al. 1993), which might lead to new specificities (Richter et al. 1995). Furthermore, even in the absence of tandemly repeated resistance genes, the repeated internal LRR structure of a single resistance gene could also undergo unequal crossing-over (Ellis et al. 1995). Such modifications would occur at a far higher frequency than mutations, allowing plants to cope with an ever-changing array of pathogens (Richter et al. 1995).

As the results presented above show that *PvH20* is tightly linked to *Co-2*, this sequence could be a useful tool to screen for the presence or the absence of this resistance gene. But, as hybridization experiments are tedious when large populations need to be analyzed, the corresponding SCAR was further characterized.

Usefulness of the SCH20 SCAR marker in the different *P. vulgaris* gene pools

A set of 24 *Co-2* or *co-2* bean genotypes, representing both Andean and Mesoamerican gene pools, were analyzed using the SCH20 SCAR marker which had been developed by Adam-Blondon et al. (1994 a). After *Dde*I restriction of the amplification product, all genotypes possessing the *Co-2* locus presented the SCAR pattern associated with resistance (0.136-kb band) (Fig. 5 A). Among the resistant genotypes were ten commercial cultivars (Ardinal to Fruidor, Fig. 5 A) and three nearly-isogenic lines for the *Co-2* gene generated independently (Co2LVE, Co2LVA and La Victoire, P12/R and P12/S). Cornell49242 was the original source of *Co-2* introgression in these cultivars through individual breeding programs. However, two susceptible Mesoamerican bean genotypes, BAT93 and TO, also presented patterns associated with *Co-2* resistance. This ambiguity remained after digestion with various other restriction enzymes (data not shown). These two genotypes display the 7-kb *Dra*I allele (Fig. 2), implying that the mutation leading to the difference between the 1.7-kb and 7-kb *Dra*I alleles lies outside the amplification

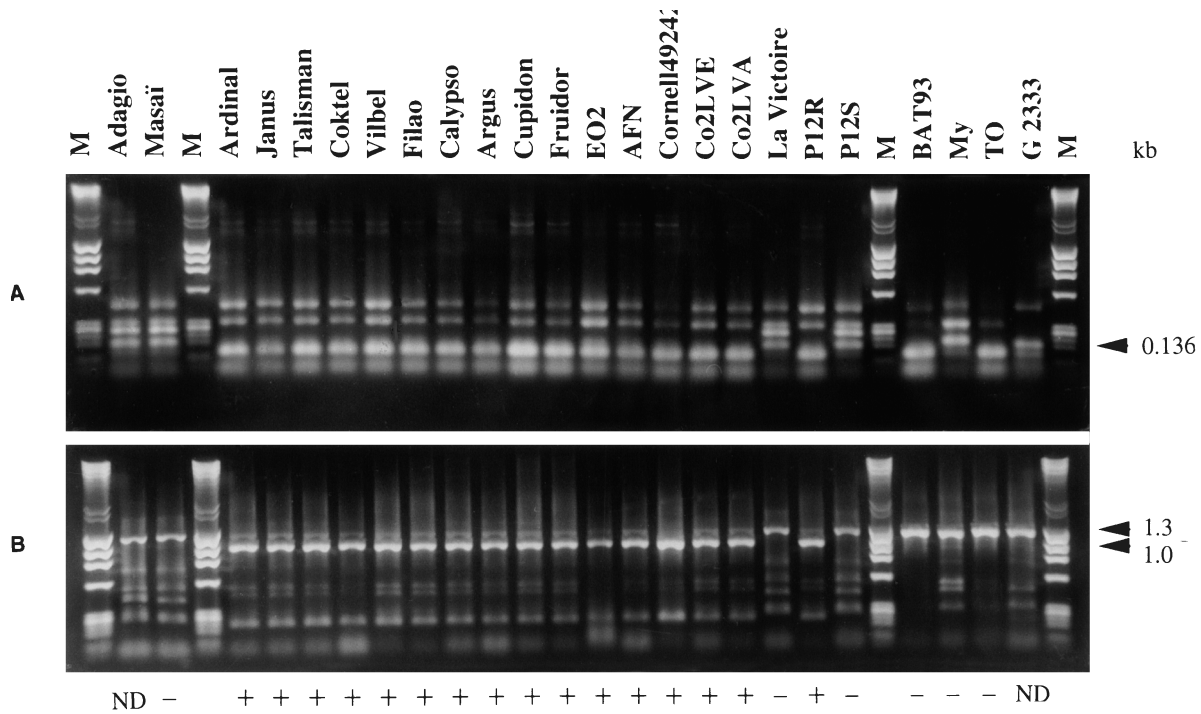

Fig. 5A, B DNA amplification of various bean genotypes with two pairs of primers. Adagio to Fruidor, AFN and La Victoire are French bean cultivars of Andean origin, Co2LVE and Co2LVA are two isolines of La Victoire possessing the *Co-2* gene, P12R and P12S are two Andean isolines diverging for the *Co-2* gene. EO2, Cornell49242, BAT93, My, TO, and G 2333 are Mesoamerican genotypes. (+) presence, (−) absence of the *Co-2* allele, (ND) not defined. *M* represents a lambda-*Hin*dIII/*Phi*X-*Hae*III marker. **A** The *PvH20* sequence was amplified using the SCH20-1 and SCH20-2 SCAR primers. The amplification product was digested by *Dde*I and separated on an agarose gel. The SCH20 SCAR marker (136 bp) is indicated by an arrow. **B** Areoli-1 and SCH20-1 primers were used to amplify a 1.3-kb band in all the genotypes tested. The amplification product was digested by *Dra*I and electrophoresis was carried out on an agarose gel. A 1.3-kb band was found to be present in *co-2* susceptible genotypes, while all *Co-2* resistant genotypes harboured a 1-kb band

product generated by the SCH20-1 and SCH20-2 primers and that there has been a recombination event between the sequence responsible for the *Co-2* specificity and the amplified sequence.

Consequently, the SCH20 SCAR marker is useful mainly in Andean germplasm. Other studies in *P. vulgaris* have already pointed out that molecular markers linked to disease resistance genes have a gene-pool-specific use (Haley et al. 1993; Miklas et al. 1993; Johnson et al. 1995). For the same reason, the SCH20 SCAR marker cannot be used in Mesoamerican germplasm, since this marker might be present in genotypes that do not possess the Mesoamerican *Co-2* resistance gene.

A PCR-based marker for the *Co-2* allele

Another primer, Areoli-1, derived from the analysis of *PvH20*-containing phage genomic clones (Creusot et al 1997) was used to carry out amplifications in combination with the SCH20-1 primer. A 1.3-kb band was amplified in all genotypes tested. After restriction of the PCR product with the *Dra*I enzyme, a polymorphism was identified (Fig. 5B). All *Co-2* resistant genotypes presented a 1-kb band whereas all *co-2* susceptible genotypes presented a 1.3-kb band (Fig. 5B). The 1-kb band (referred to as SCAreoli) co-segregated with the SCH20 SCAR marker in the backcross progeny between EO2 and Corel (Fig. 3A). Interestingly, whereas BAT93 and JaloEEP558 both carry the *co-2* susceptible allele, and therefore exhibit a 1.3-kb band after *Dra*I digestion, BAT93 did not display any digestion product (Fig. 5B) whereas JaloEEP558 revealed a more complex digestion pattern including the 1.3-kb and four other smaller bands (data not shown), similar to those observed for P12S, La Victoire and Masaï (Fig. 5B). These polymorphic bands were scored in the RILs derived from the cross between BAT93 and JaloEEP558 and co-segregated with the SCH20 SCAR marker. These findings supported the idea that there is a family of related sequences in the vicinity of the *Co-2* locus.

Two genotypes, Adagio and G 2333, known to possess a resistance gene(s) against anthracnose, were also included in our experiments. Adagio is a French commercial breeding line, said to be "resistant against anthracnose". For French breeders "anthracnose resistant" is often synonymous with "possesses *Co-2*", since *Co-2* is the only resistance gene known to have been introduced in French bean breeding programs. However, being resistant to a strain avirulent towards *Co-2* does not provide undisputed evidence that Adagio harbours *Co-2*. Adagio resistance might be mediated by

another resistance gene. Our results suggest that Adagio does not possess the *Co-2* resistance gene (Fig. 5 B), since it presents the 1.3-kb band, unless a recombination has occurred between the 1.3-kb sequence and the actual *Co*-2 sequence. However, an allelism test between Adagio and Cornell49242 will provide unequivocal results. G 2333 is also an important source of anthracnose resistance since no race of the pathogen has been reported as virulent on this genotype (Pastor-Corrales et al. 1994). Genetic analysis has revealed that G 2333 harbours at least three resistance genes, one of which should be *Co-5* (Young and Kelly 1996 a). The presence of multiple resistance genes may thus account for its broad-spectrum resistance. The identification of individual resistance genes by classical genetic analysis is difficult, since each resistance gene must be isolated through backcrossing in separate lines. However, molecular markers closely linked to known resistance genes might be useful to speed up the genetic dissection of durable resistance. In this regard, our results indicated that G 2333 may not harbour *Co-2* (Fig. 5 B).

The SCAreoli SCAR molecular marker described here will be useful in MAS strategies for *Co-2* introgression, since it is not gene-pool specific. It can be used in combination with other markers linked to anthracnose resistance genes (Young et Kelly 1996 a) or with markers linked to resistance genes against other pathogens such as *BCMV* (Melotto et al. 1996; Johnson et al. 1997) or rust (Haley et al. 1994). DNA markers can also be used in a more fundamental way, to initiate a map-based cloning strategy. Cloning of disease resistance genes constitutes an exciting breakthrough for understanding the molecular basis of plant disease resistance. A map-based cloning approach of the *Co-2* resistance gene is underway (Creusot et al. 1997).

Acknowledgments We are grateful to D. O'Sullivan for critical reading of the manuscript. We thank R. Boyer for photographic work. The research was supported by CNRS, INRA (AIP 94–4920), and the Ministère de l'Education Nationale, Paris.

References

Adam-Blondon AF, Sévignac M, Bannerot H, Dron M (1994 a) SCAR, RAPD and RFLP markers linked to a dominant gene (*Are*) conferring resistance to anthracnose in common bean. Theor Appl Genet 88 : 865–870

Adam-Blondon AF, Sévignac M, Dron M (1994 b) A genetic map of common bean to localize specific resistance genes against anthracnose. Genome 37 : 915–924

Bannerot H (1965) Résultats de l'infection d'une collection de haricots par six races physiologiques d'anthracnose. Ann Amélior Plant 15 : 201–222

Bannerot H, Derieux M, Fouilloux G (1971) Mise en évidence d'un second gène de résistance totale à l'anthracnose chez le haricot. Ann Amélior Plant 21 : 83–85

Barrus MF (1911) Variation in varieties of beans in their susceptibility to anthracnose. Phytopathology 1 : 190–195

Bennetzen J, Richter T, Hu G, SanMiguel P, Hong K, Frederik R, Hulbert S (1994) Organization and hyperevolution of rust resistance genes in maize. In: MJ Daniels et al. (eds) Advances in molecular genetics of plant-microbe interactions vol 3. Kluwer Academic Publishers, Netherlands, pp 261–266

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1994) *RPS*2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265 : 1856–1860

Cai D, Kleine M, Kifle S, Harloff HJ, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMJ, Lange W, Stiekema WJ, Wyss U, Grundler FMW, Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275 : 832–834

Creusot F, Geffroy V, Franco De Oliveira J, Macadré C, Sévignac M, Adam-Blondon AF, Ferrier Cana E, Thoquet P, Riou C, Cohen S, Dron M, Langin T (1997) Resistance in *Phaseolus vulgaris*: identification of DNA sequences linked to resistance loci against *Colletotrichum lindemuthianum*. In: Plant disease resistance gene function, EMBO Workshop Maratea, Italy, May 18–20, p 11

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for VAX. Nucleic Acids Res 12 : 387–395

Dickinson MJ, Jones DA, Jones JDG (1993) Close linkage between the *Cf-2*/*Cf-5* and *Mi* resistance loci in tomato. Mol Plant-Microbe Int 6 : 341–347

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JDG (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84 : 451–459

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19 : 11–15

Ellis JG, Lawrence GJ, Finnegan EJ, Anderson PA (1995) Contrasting complexity of two rust resistance loci in flax. Proc Natl Acad Sci USA 92 : 4185–4188

FAO (1980) Food balance sheets: 1975–1977 average and per capita food supplies, 1961–1965 average, 1967–1977. Food and Agriculture Organization, Rome

Flor HH (1955) Host-parasite interaction in flax rust. Its genetics and other implications. Phytopathology 45 : 680–685

Fouilloux G (1976) L'anthracnose du haricot (*Colletotrichum lindemuthianum*, Sacc. et Magn.): nouvelles sources de résistance et nouvelles races physiologiques. Ann Amélior Plant 26 : 443–453

Fouilloux G (1979) New races of bean anthracnose and consequences of our breeding programs. In: Maraite H, Meyer JA (eds) Diseases of tropical food crops. Université catholique de Louvain-la-neuve, Belgium, pp 221–235

Freyre R, Johnson WC, Shirmohamadali A, Llaca V, Tsai SM, Pereira PA, Skroch P, Nienhuis J, Adam-Blondon AF, Dron M, Gepts P (1996) Correlation among RFLP maps in common bean. Annu Rep Bean Improv Coop 39 : 231–232

Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, Sattler A, Innes RW, Dangl JL (1995) Structure of the *Arabidopsis RPM*1 gene enabling dual-specificity disease resistance. Science 269 : 843–846

Haley SD, Miklas PN, Stavely JR, Byrum J, Kelly JD (1993) Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theor Appl Genet 86 : 505–512

Haley SD, Afanador LK, Miklas PN, Stavely JR, Kelly JD (1994) Heterogenous inbred populations are useful as sources of near-isogenic lines for RAPD marker localization. Theor Appl Genet 88 : 337–342

Johnson E, Miklas PN, Stavely JR, Martinez-Cruzado JC (1995) Coupling- and repulsion-phase RAPDs for marker-assisted selection of the PI 181996 rust resistance in common bean. Theor Appl Genet 90 : 659–664

Johnson WC, Guzmán P, Mandala D, Mkandawire ABC, Temple S, Gilbertson RL, Gepts P (1997) Molecular tagging of the *bc-3*

gene for introgression into the Andean common bean. Crop Sci 37:248–254
Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789–793
Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750
Kelly JD, Young RA (1996) Proposed symbols for anthracnose resistance genes. Annu Rep Bean Improv Coop 39:20–24
Kelly JD, Afanador L, Cameron LS (1994) New races of *Colletotrichum lindemuthianum* in Michigan and implications in dry bean resistance breeding. Plant Dis 78:892–894
Kobe B, Deisenhofer J (1994) The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 19:415–421
Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175
Lander ES, Green P, Abrahamson J, Barlow A, Daly M, Lincoln ES, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural population. Genomics 1:174–181
Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7:1195–1206
Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432–1436
Martin GB, Frary A, Wu T, Brommonschenkel S, Chunwongse J, Earle ED, Tanksley SD (1994) A member of the tomato *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death. Plant Cell 6:1543–1552
Mastenbroek C (1960) A breeding programme for resistance to anthracnose in dry shell haricot beans, based on a new gene. Euphytica 9:177–184
McRostie GP (1919) Inheritance of anthracnose resistance as indicated by a cross between a resistant and a susceptible bean. Phytopathology 9:141–148
Melchinger AE (1990) Use of molecular markers in breeding for oligogenic disease resistance. Plant Breed 104:1–19
Melotto M, Afanador L, Kelly JD (1996) Development of a SCAR marker linked to the *I* gene in common bean. Genome 39:1216–1219
Michelmore R (1995) Molecular approaches to manipulation of disease resistance genes. Annu Rev Phytopathol 15:393–427
Miklas PN, Stavely JR, Kelly JD (1993) Identification and potential use of a molecular marker for rust resistance in common bean. Theor Appl Genet 85:745–749
Mindrinos M, Katagiri F, Yu GL, Ausubel FM (1994) The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and a leucine-rich repeat. Cell 78:1089–1099
Mundt CC (1990) Probability of mutation to multiple virulence and durability of resistance gene pyramids. Phytopathology 80:221–223
Nelson RR (1978) Genetics of horizontal resistance to plant diseases. Annu Rev Phytopathol 16:359–378
Nodari RO, Tsai SM, Gilbertson RL, Gepts P (1993) Towards an integrated linkage map of common bean. 2. Development of an RFLP-based linkage map. Theor Appl Genet 85:513–520
Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R (1997) The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide-binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521–532
Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993
Parker JE, Coleman MJ, Szabo V, Frost LN, Schmidt R, vander-Biezen EA, Moores T, Dean C, Daniels MJ, Jones JDG (1997) The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and *L6*. Plant Cell 9:879–894
Pastor-Corrales MA, Tu JC (1989) Anthracnose. In: Schwartz HF, Pastor-Corrales MA (eds) Bean production problems in the tropics. Centro International de Agricultura Tropical, Cali, Colombia, pp 77–104
Pastor-Corrales MA, Erazo OA, Estrada EI, Singh SP (1994) Inheritance of anthracnose resistance in common bean accession G 2333. Plant Dis 78:959–962
Pryor T (1987) The origin and structure of fungal disease resistance genes in plants. Trends Genet 3:157–161
Pryor T, Ellis J (1993) The genetic complexity of fungal resistance genes in plants. Adv Plant Pathol 10:281–305
Reed KC, Man DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 13:7207–7221
Richter TE, Pryor TJ, Bennetzen JL, Hulbert SH (1995) New rust resistance specificities associated with recombination in the *Rp1* complex in maize. Genetics 141:373–381
Singh SP, Nodari R, Gepts P (1991) Genetic diversity in cultivated common bean. I. Allozymes. Crop Sci 31:19–23
Sudupak MA, Bennetzen JL, Hulbert SH (1993) Unequal exchange and meiotic instability of disease-resistance genes in the *Rp1* region of maize. Genetics 133:119–125
Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of tobacco mosaic virus resistance gene *N*: similarity to *Toll* and *interleukin*-1 receptor. Cell 78:1101–1115
Young RA, Kelly JD (1996 a) Characterization of the genetic resistance to *Colletotrichum lindemuthianum* in common bean differential cultivars. Plant Dis 80:650–654
Young RA, Kelly JD (1996 b) RAPD markers flanking the *Are* gene for anthracnose resistance in common bean. J Am Soc Hort Sci 121:37–41
Yu YG, Buss GR, Saghai Maroof MA (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11751–11756