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Construction of near-isogenic lines to investigate the efficiency of different resistance genes to anthracnose

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Abstract A suitable experimental model was designed with the aim of investigating the specific effect of different resistance genes in the *Phaseolus vulgaris – Colletotrichum lindemuthianum* interaction. The four resistance genes examined were chosen because they confer a different phenotype (resistance or susceptibility) to the lines carrying them when challenged by a range of *C. lindemuthianum* races. These different resistance genes were introgressed independently into the same susceptible recipient line. The isogenicity of the five near-isogenic lines (NILs) thus obtained (four resistant lines, one susceptible line = recipient line) was assessed by a RAPD analysis. The hypersensitive reaction occurred at the same time after infection, whatever the resistance gene present, when the NILs were challenged by the avirulent race 9 of the pathogen. In contrast, the pathogen development was arrested more or less rapidly in the different NILs. At the first stages of the infection process, the transcripts encoding phenylalanine ammonia-lyase were accumulated to a different extent in the different resistant NILs but always to a higher level than in the susceptible recipient line. These results suggest that the different resistance genes operate through more than one way in the production of defense factors.

Key words *Phaseolus vulgaris* · *Colletotrichum lindemuthianum* · PAL mRNA · Cytology · RAPD

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Introduction

The establishment of plant/pathogen interactions involves a complex interplay between the two partners. A successful infection (basic compatibility) requires a range of pathogen functions that allow it to withstand the natural defense mechanisms of the plant (general resistance). The race/cultivar specificity is superimposed on the basic compatibility. For more than 40 different interactions, it has been demonstrated, or suggested, that the race/cultivar specificity is controlled by the interaction (direct or indirect) of the products of single genes in each partner: a resistance gene in the plant and an avirulence gene in the pathogen (Gabriel and Rolfe 1990). The interaction between a plant cultivar harboring a resistance gene and a race of the pathogen carrying the complementary avirulence gene leads to resistance; this interaction has been designated incompatible. Absence or inactivation of either the resistance gene or the matching avirulence gene results in the development of disease; the interaction is compatible. Resistance is usually characterized by the so-called hypersensitive response which corresponds to the formation of necrotic lesions at the site of pathogen entry and to the restriction of pathogen growth and spread.

Plants respond to infection by mobilizing a complex network of active defense mechanisms. These include the generation of activated oxygen species (Williamson and Scandalios 1993), modification of cell-wall structure, accumulation of compounds exhibiting antimicrobial activity when tested in vitro (phytoalexines, some PR proteins), and the synthesis of proteins whose functions are still unknown (Bowles 1990). Most of these defense responses involve the transcriptional activation of the corresponding genes (reviewed in Dixon and Harrison 1990).

It has been shown that different time-courses in the mRNA induction of defense genes can be triggered by different compatible races of the pathogen along with different kinetics of pathogen development in plant

tissues (Mahé et al. 1992b). It is likely that similar results would be observed if different cultivars were challenged by the same race of the pathogen. The results of O'Connell and Bailey (1988), showing that the development of a given race of *C. lindemuthianum* was arrested more or less rapidly in different bean cultivars, supports this assumption. Thus, to avoid misleading conclusions, plant and pathogen backgrounds have to be controlled when specific race-cultivar interaction (determined by the gene-for-gene interaction) is studied.

In our laboratory, we are interested in the interaction between bean (*Phaseolus vulgaris*) and the fungus *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease. In bean, several resistance genes to anthracnose have been identified by classical genetics (Fouilloux 1979). Do these different resistance genes determine the restriction of the fungus growth with the same efficiency and do they allow the activation of defense mechanisms with the same kinetics?

In order to compare the individual effect of different resistance genes without interference from the genetic background in which they have been selected, we have constructed near-isogenic lines (NILs) each of which carry a different resistance gene. Four disease-resistance genes were incorporated into the same susceptible variety by crossing with stocks that carry the resistance gene(s). The resistance spectrum given by the four selected resistance genes is summarized in Table 1. To assess the isogenicity of the constructed NILs, DNA fingerprinting was performed using the random amplified polymorphic DNA (RAPD) analysis technique (Williams et al. 1990). This allowed us to screen efficiently for the allelic status of different loci throughout the genome and thus to determine the degree to which the genomic content of the donor has been conserved.

The efficiency of the different resistance genes was examined by performing cytological analyses, in order to study the progression of the fungus within the host tissues, and by investigating the time-course of the PAL defense gene mRNA. Activation of the gene encoding phenylalanine ammonialyase, PAL (the first enzyme of the phenylpropanoid pathway, involved in the synthesis of lignin and phytoalexins in some plant species), was considered since, in most cases, it is activated earlier in the incompatible interaction than in the compatible one and seems to be correlated with race-specific resistance (Ralton et al. 1988; Lee et al. 1992; Mahé et al. 1993).

Table 1 Phenotype conferred by the different resistance genes studied when confronted with different races of *C. lindemuthianum*

Race no.	Resistance genes[a]			
	Mex1a	*Mex1b*	*Mex2*	*ARE*
1	S	S	R	R
9	R	R	R	R
21	R	S	R	S
31	R	S	R	R
36	R	S	S	R
79	R	R	R	S
82	R	S	S	S
88	S	S	R	S

[a] S, susceptibility; R, resistance

Material and methods

Plant material

We used a collection of four breeding lines resistant to anthracnose, AFN, EO2, My and Mz, and one susceptible line, La Victoire (LV): seeds were obtained from INRA (Versailles, France). AFN carries the *ARE* and *Mex2* resistance genes, EO2 carries the *ARE* and *RVI* resistance genes, My and Mz carry respectively the *Mex1a* and *Mex1b* resistance genes. These genes are genetically independent except that the last two are either very closely genetically linked or else are alleles of the same gene (Fouilloux 1979).

Plants were grown in a greenhouse compartment at 24 °C under a photoperiod of 16 h light/8 h dark and a relative humidity at 70%.

Fungal material

Race 9 of *C. lindemuthianum*, which is avirulent towards the four resistance genes studied, was used for infection tests. It was provided by F. Legrand and J. Tailler (Université Paris XI, Orsay, France) and was grown as described in Gantet et al. (1991).

Pathogenicity tests

In the course of the construction of the NILs, in vitro pathogenicity tests were performed as described by Gantet et al. (1991).

For cytological and Northern-blot analyses, plants were infected as described previously (Mahé et al. 1992a).

Cytology

Pieces of leaves from the five NILs were collected at different times after infection (24h, 37h, 48h, 61h, 73h, 85h, 97h, 109h, 121h) and analyzed as described by Mahé et al. (1992a).

Molecular analysis

Plant genomic DNA was isolated from leaves basically as described by Dellaporta et al. (1983) except that DNA samples were purified by cesium-chloride gradients (100 000 rpm for 4 h in a TLN100 Beckman rotor). For PCR amplification (RAPD) oligonucleotide primers (10-mers) were puchased from Operon Technologies (Alameda, Calif.). The reactions were performed as described by Williams et al. (1990) in a Braun thermocycler using *Taq* DNA polymerase from Appligene (Strasbourg, France). According to the primer, annealing temperatures were 35° or 42 °C. Amplification products were analyzed by electrophoresis on 1.4% agarose gels and detected by staining with ethidium bromide.

Total RNA was isolated from leaves harvested at the same times as indicated for the cytological analysis and Northern blotting was performed as in Mahé et al. (1992 a). The Northern blots were successively hybridized with a bean PALcDNA probe (pPAL5; Edwards et al. 1985) and a bean EF-1α cDNA probe (pCH0041; Axelos et al. 1989). The autoradiograms were scanned by means of a Canon CLC-10 scanner and analyzed using Image 1.43 software from Macintosh. To quanitify the PAL transcript levels, the scanning values were normalized with respect to the constitutively expressed EF-1α mRNA as described in Mahé et al (1992a).

Results

Construction and verification of the near-isogenic lines

A cross between each source of the resistance genes and the susceptible variety (recurrent parent) was made. Hybrids resistant to *C. lindemuthianum* race 9 were selected in the progeny and backcrossed to the recurrent parent. The progeny was screened for resistance and crossed again to the recurrent parent and the process repeated. Table 2 summarizes the steps undergone to obtain the near-isogenic lines (NILs). The homozygous genotypes at the resistance-gene locus, either resistant (YR, ZR, AR, ER) or susceptible (YS, ZS, AS, ES). were then selected after selfing. Using discriminant races for pathogenicity tests, it was checked that the *ARE* gene and the *RVI* gene, initially present in AFN and EO2 respectively, were no longer present in the NILs arising from them.

To assess the isogenicity of the selected NILs, 30 different random sequence primers were used to amplify genomic DNA isolated from these NILs and from the parent lines. Polymorphism was identified as differences in banding patterns between the different lines. Figure 1 shows the results of such an experiment for a single primer. The patterns obtained using DNA from the different donor lines (My, Mz, AFN, EO2) exhibit polymorphism when compared to the recurrent line (LV). In contrast, the patterns of the different NILs were identical among themselves and identical to that of LV.

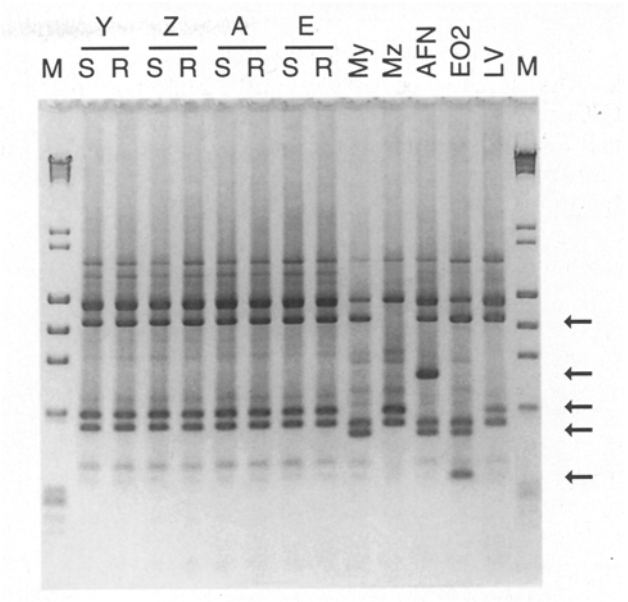

Fig. 1 PCR amplification of bean genomic DNA with a 10-mer primer. DNA from the parental genotypes (My, MZ, AFN, EO2 and LV) and from the NILs (YS and YR, ZS and ZR, AS and AR, ES and ER) arising from them, as described in Table 2, was amplified using the G5 primer (CTGAGACGGA) from OPERON (Alameda, Calif.) (Materials and methods). Amplification products were resolved by electrophoresis on a 1.4% agarose gel. *M* molecular-weight markers (mix of Lambda/*Hin*dIII and ΦX174/*Hae*III DNA fragments). *Arrows* point to polymorphic bands present in the donor lines

Table 2 Scheme of construction of the NILs

Donor line Resistance genes	My *Mex1a*	Mz *Mex1b*	AFN *MexII+ARE*	EO2 *ARE+RIV*
Number of backcrosses to the susceptible line LV	5	7	6	8
Number of selfings Resulting NILS	4 YS/YR	3 ZS/ZR	3 AS/AR	4 ES/ER

The results of the polymorphism analysis between each donor line and the recipient line are reported in Table 3. For each primer tested, by comparison to LV, bands polymorphic for at least one donor line were scored. An example of scored bands (indicated with arrows) is given in Fig. 1. Loci with known genetic locations were recorded (whether or not polymorphic in the donor lines compared to LV). As the EO2 genotype has already been used to generate a genetic map of bean (Adam-Blondon et al. 1994), it helps us to correlate some of the bands to already mapped loci. The genotype for each of the donor parent at these loci is schematized in Fig. 2. This figure shows that the polymorphism was screened throughout the genome and reveals the different genetic content for each donor line and for LV.

NILs were typed for all the parental polymorphic bands (whether or not mapped). No difference between the NILs were found and except in two cases all exhibited the genotype from the recurrent parent (LV). The first exception concerns the ROH20 locus (revealed by the primer H20) which mapped 1 cM from the ARE resistance gene. When compared to LV, EO2 and AFN (which both carry the ARE gene) are polymorphic at this locus (Fig. 2). The corresponding allele is also present in

Table 3 Screening of the polymorphism observed when the four donor lines were compared to the recipient line (LV)

Observed polymorphism	My	Mz	AFN	EO2
Polymorphic bands within all the scored bands	90/129 69.8%	61/124 49.2%	22/125 17.6%	97/129 75.2%
Polymorphic bands within the mapped loci	25/64 60.9%	32/64 50%	9/64 15%	11/64 82.8%

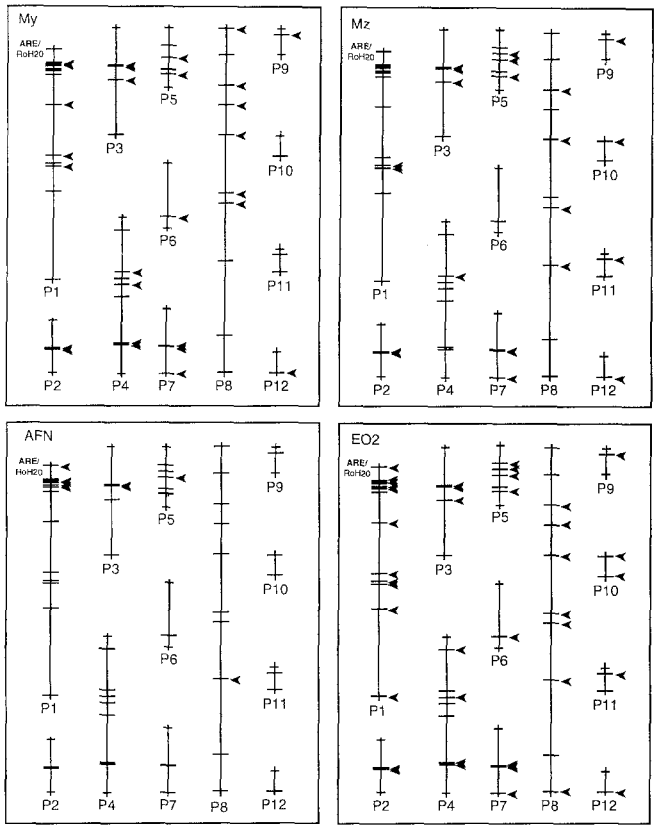

Fig. 2 Genetic map of the polymorphic loci identified between the four donor parents My, Mz, AFN, EO2 and the recipient line LV. The 12 linkage groups (*P1* to *P12*) are defined according to Adam-Blondon et al. (1994). The *horizontal lines* locate the different RAPD markers tested. The polymorphic loci are denoted by a *black triangle*

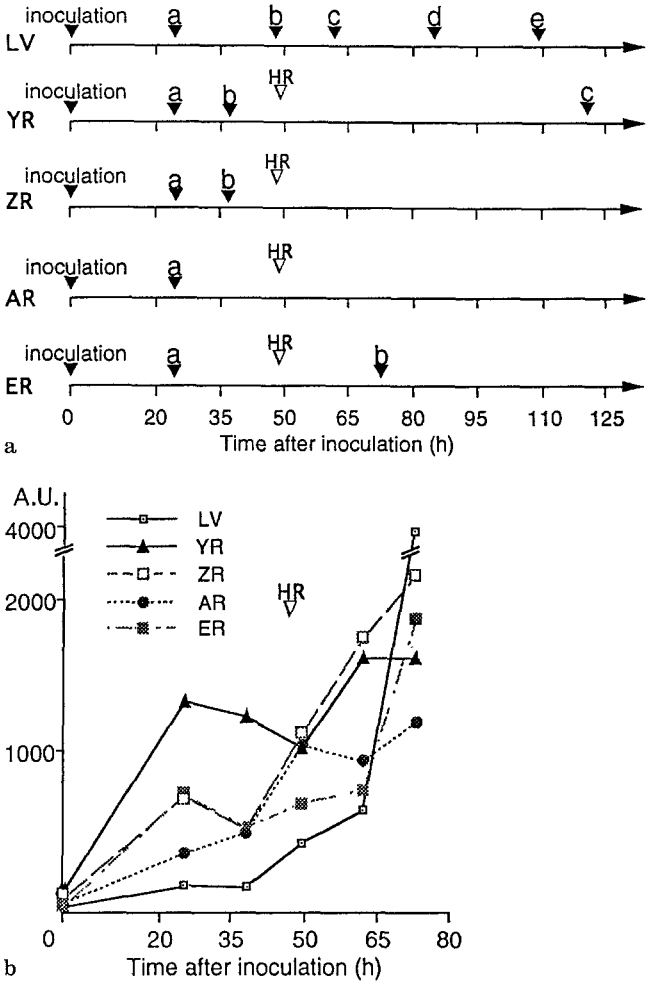

Fig. 3 Time course of pathogen development (**a**) and PAL mRNA accumulation (**b**) in bean NILs infected with race 9 of *C. lindemuthianum*. **a** Cytological observations are *a* appressorium; *b* primary hyphae emerging from infection vesicles; *c* developed primary hyphae in the first infected cell; *d* extensive development of primary hyphae; *e* well developed secondary hyphae. **b** PAL transcript levels were measured at the indicated times and normalized as described in Material and methods. *A.U.* arbitrary units; *HR* hypersensitive reaction

the resistant NILs (ER and AR) arising from these cultivars although, in the case of the line issuing from AFN, the ARE gene is no longer present. The second polymorphic band has not yet been mapped and was present both in My and in the YR line arising from it. A morphological marker *Anp* located at about 2.3 cM from the *Mex2* resistance gene (Gantet et al. 1991) and present in AFN was not found in the *R* line arising from the introgression of *Mex2* from AFN.

Fungal development and PAL transcript level determination after infection of NILs

After infection of the four resistant NILs, YR, ZR, AR, and ER, with the avirulent race 9 of *C. lindemuthianum*, the hypersensitive reaction (HR) was visible at the same time (48 h after infection) whatever the resistance gene present. Nevertheless, fungus development was arrested more or less rapidly (Fig. 3a). For example, in the presence of *Mex1a* (harbored by YR) the fungus was able to develop until the formation of primary hyphae within the infected cell (stage c, Fig. 3a) whereas *Mex2* (carried by AR) seemed to confer the best efficiency in restricting the fungus since no fungal intracellular structure was observed. An intermediate situation was encountered for the *Mex1b* and *ARE* genes (Fig. 3a). In the case of the *ARE* gene, the present results differ from previous ones obtained using a different bean genetic background, the P12R/P12S NILs (Mahé et al. 1993). Thus, in ER, race 9 develops until the formation of infection vesicles with minute hyphae emerging from them (stage b, Fig. 3a) whereas in P12R no fungal intracellular structure is observed. It should also be noted that after the HR response has occurred, pathogen development was slowed down and that, whatever the resistance gene present, the fungus was always restricted within the infected cell.

In the compatible interactions, the time-course of the pathogen development was similar in LV and in the four susceptible isogenic lines, YS, ZS, AS, ES. Therefore, only the results obtained with LV are reported. Race 9 of

C. lindemuthianum developed slightly slower when compared to previous experiments involving P12S and race 9 (Mahé et al. 1992a). In this latter case, the appearance of well-developed secondary hyphae (stage e, Fig. 3a) was observed 18 h earlier.

To study PAL transcript accumulation in bean after infection by *C. lindemuthianum*, Northern blots of total RNA were hybridized to a cloned PAL cDNA. Previous results (Bell et al. 1986; Mahé et al. 1993) have shown that, in incompatible interactions, the PAL transcript starts to accumulate at the time of pathogen penetration into epidermal cell layers and before the hypersensitive reaction is observed. Therefore, we have focused our investigation on the first 60 h following infection, before the onset of PAL mRNA accumulation in the susceptible line. As expected, the amount of PAL transcript was higher in the incompatible interactions compared to the compatible interaction (LV/race 9) during the period examined (Fig. 3b). The level of accumulation differed according to the interaction studied, the highest (about 8.5-fold) was for YR/race 9 (interaction allowing the fungal growth to progress the most) and the lowest (2–3-fold) in AR (the line that was also the most efficient in arresting pathogen growth).

Discussion

While the extent of polymorphism is relatively high between the different donor genotypes and the recipient line (LV), a very low residual polymorphism has been detected between the NILs and LV. It has been estimated that the reconstruction of the recurrent parent genotype requires more than six generations with backcross breeding (Tanksley et al. 1989). However, it is likely that small structural differences still exist between the different genotypes; for example, some linkage drag (Zeven et al. 1983) may occur in the region of the targeted genes. Here, the size of the donor DNA linked to the different resistance genes could not be estimated as three of these resistance genes have not yet been mapped in the genome of bean. Nevertheless, in the case of the *ARE* gene, mapped at the end of linkage group number one (Adam-Blondon et al. 1994), the nearest informative marker tested is located at 13 cM on one side, suggesting that the introgressed fragment may lie at less than 13 cM on this side; similarly, on one side of the *Mex2* gene, the morphological marker *Anp* allowed us to locate the boundary of the introgressed fragment at less than 2.3 cM.

The low level of diversity displayed by the NILs allowed an accurate comparison of the effects of the different resistance genes on the disease response. Nevertheless, it is clear that to fully understand the mode of action of disease-resistance genes, bean transgenic lines for a resistance gene should be constructed in order to obtain truly isogenic lines.

After infection of the resistant NILs with race 9 of *C. lindemuthianum*, the HR was observed at the same time after infection whatever the resistance gene present. Yet, the growth of the fungus was not arrested at the same stage of development, though in the four cases, the pathogen was restricted within the penetrated epidermal cells.

The time-course of PAL transcript accumulation was investigated in the infected NILs, during the period of time surrounding the observation of the HR. In accordance with previously published results (Mahé et al. 1993), PAL transcripts reached a higher level in the four resistant NILs when compared to the susceptible line. They accumulated in a greater amount in the presence of the *Mex1a* resistance gene which seems the less effective in preventing the fungus growth. In contrast, they were only slightly induced in the presence of the most effective resistance gene of our model (*Mex2*). As a close correlation between the accumulation of PAL mRNA and increased enzyme synthesis and activity has been reported (Bell et al. 1986), these observations raise the question of the involvment of PAL gene expression in the mechanism of inhibition of pathogen growth. PAL gene activation might only be a consequence of the establishment of resistance.

Some differences were obvious when the compatible interactions LV-race 9 (this paper) and P12S-race 9 (Mahé et al. 1993), on one hand, and the incompatible interactions ER (*ARE*)-race 9 (this paper) and P12R (*ARE*)-race 9 (Mahé et al. 1993), on the other hand, were compared. As the same race of the pathogen was used for infection and the same resistance gene (*ARE*) was involved, the differences observed in the time-course of pathogen development and PAL mRNA accumulation cannot be ascribed to differences in the virulence of the pathogen. These differences might rather result from a different capacity of the plant backgrounds for defense, as already shown by the experiments of O'Connell and Bailey (1988). In this regard, it would be interesting to estimate the genetic relatedness between the P12 NILs and the NILs constructed from LV. As pointed out by Briggs and Johal (1994), in plant-pathogen interactions, "the host trait of resistance to infection and the parasite trait of infectiousness are controlled by the combined genotypes of host and parasite rather than by either genotype alone". This is true in the context of basic compatibility as well as of specific resistance and underlines the requirement of using isogenic lines to study the specific effect of different resistance genes.

Our results show that in closely related genetic backgrounds, different resistance genes induce plant resistance with different efficiencies. Although we cannot exclude the possibility that the differences observed were attributable to some residual lack of isogenicity, it is likely that the different resistance genes do not operate in a univocal way.

The activity of the resistance genes is pleiotropic, they affect the expression of several structural genes. Several distinct post-infectional mRNA species are differently regulated by the resistance genes but it is not known whether these responses are the cause, or merely a

symptom of resistance. Given the variety of metabolic pathways activated, a regulatory role for the resistance loci can be postulated. The *Pto* gene from tomato that confers resistance against *Ps syringae* pv tomato, probably encodes a serine/threonine protein kinase (Martin et al. 1993). This finding supports the idea that a signal transduction mechanism is required for the expression of resistance and it can be postulated that different resistance genes should be active at different steps of this transduction pathway, or else activate different transduction pathways.

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