Molecular Physiology and Genetics of Coffee Resistance to Parasites

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SUMMARY

Coffea arabica varieties usually display a high yielding and good coffee quality, but exhibit a high susceptibility to many pests and diseases. Enhancing their resistance to parasites have become a crucial priority toward an economic and sustainable coffee production. Research activities were therefore developped to identify and clone genes involved in the specific resistance of coffee to nematodes (*Meloidogyne* spp.) and rust (*Hemileia vastatrix*).

A positional cloning project was started to isolate a resistance gene derived from *C. canephora*, that confers resistance against *M. exigua*. Based on the analysis of progenies obtained from resistant introgressed arabica lines, 15 AFLP markers tightly linked to the resistance gene were identified. Further linkage investigations allowed the construction of a localised genetic map of the chromosome segment carrying the *M. exigua* resistance. The low rate of recombination indicated these markers could be useful landmarks for map-based cloning of the resistance gene. With this purpose, a BAC library is being constructed.

In addition, disease resistance gene analogs (RGA) were cloned in coffee (*C. arabica* and *C. canephora*) using DNA primers designed from conserved motifs (NBS) of known plant resistance (R) genes. Analysis of PCR-derived coffee NBS sequences revealed nine distinct families of RGAs, belonging to the non-TIR class type of R-genes, in both species. Sequence variation observed among coffee RGAs suggested point mutations as the primary source of diversity within RGAs families. Efforts are being pursued to explore the possibility of implication of isolated coffee RGA families in the identified nematode resistance.

In parallel, genes early expressed during the specific resistance reaction of coffee (*C. arabica*) to the fungus *H. vastatrix* were isolated from cDNA libraries constructed using the suppression subtractive hybridization method (Diatchenko et al., 1996). cDNAs clones showing specific expression in the early stages of the resistance reaction were selected and characterized. Similarities were found with plant sequences involved in plant defence reactions such as chitinases, heat shock proteins, cytochroms P450, metallothioneins and ionic channels. Further work will aim at understanding the role of selected clones in the mechanism of specific coffee resistance to parasites.

INTRODUCTION

Coffea arabica varieties usually display a high yielding and good coffee quality, but exhibit a high susceptibility to many pests and diseases like leaf rust (*Hemileia vastatrix*, Berk & Br.),

coffee berry disease (*Colletotrichum kahawae*), coffee berry borer (*Hypothenemus hampei*), stem borer (*Xylotrechus quadripes* Chevr.) and nematodes (*Meloidogyne spp.* and *Pratylenchus spp.*). *C. canephora* (Robusta coffee) is more tolerant to these diseases and pests but product quality is considered by the consumers as rather inferior. Enhancing arabica resistance to parasites has become a crucial priority toward an economic and sustainable coffee production. Hence, transfer of desirable genes in particular for disease resistance from diploid species like *C. canephora* and *C. liberica* into tetraploid arabica cultivars without affecting quality traits has been the main objective of arabica breeding.

Molecular techniques are valuable tools for improving the efficiency of conventional coffee breeding by allowing indirect selection for resistance by looking for molecular markers linked to that resistance trait (Lashermes et al., 2000a). Such strategy is especially useful when pathogenicity tests are time-consuming, often destructive for the progenies to evaluate, and difficult to interpret. Availability of tightly linked genetic markers for resistance genes are of great help in selecting plants carrying these genes without subjecting them to pathogen attacks.

In addition, molecular data derived from the sequencing of model plant genomes and from plant Expressed Sequence Tags (ESTs) projects provide a growing body of knowledge that is expected to bring up the research on coffee more pertinent and efficient. For instance, the recent cloning of genes for resistance (R) against diverse pathogens from a variety of plants has revealed that many share conserved sequence motifs (Ellis et al., 2000). This provides the possibility of isolating numerous additional resistance genes (i. e. Resistance Gene Analogs or RGA) by polymerase chain reaction (PCR) with degenerate oligonucleotide primers.

Finally, with the use of molecular techniques, it is now possible to facilitate the transfer of desirable genes among plant varieties. Coffee genetic transformation has been successfully achieved by several research groups (Hatanaka et al., 1999; Leroy et al., 1999; Spiral et al., 1999). Our research activities were therefore developped with the aim of identifying and cloning the genes that are involved in the specific resistance of coffee to parasites. We present here some results we obtained on coffee RGAs, as well as on nematodes (*Meloidogyne* spp.) and rust (*Hemileia vastatrix*) resistance genes in coffee.

RGAs

Numerous disease resistance gene analogs (RGA) were cloned in coffee using DNA primers designed from conserved motifs (NBS) of known genes of plant resistance (R). Plant material involved accessions of both species *C. arabica* and *C. canephora*. Genomic DNA as well as mRNA extracted from leaves were used. Origin, diversity and evolution of NBS-type disease-resistance genes in coffee trees were investigated (Noir et al., 2001). Efforts are being pursued to explore the possibility of implication of isolated coffee RGA families in the identified specific resistances to parasites.

NEMATODE RESISTANCE GENES

A positional cloning project was started to isolate a resistance gene derived from *C. canephora*, that confers resistance against *M. exigua* (Bertrand et al., 2001). In particular, efforts were directed to the identification of AFLP (amplified fragment length polymorphism) markers associated with the gene *Mex-1* conferring the resistance to *M. exigua*.

The AFLP procedure was performed essentially as described by Vos et al. (1995) with minor adaptations for coffee DNA (Lashermes et al., 2000b). An aliquot of 500 ng genomic DNA

was digested with restriction enzymes *Eco*R1 and *Mse*I. Restriction fragments were ligated with double-strand *Eco*RI and *Mse*I-adapters. A total of 342 primer combinations derived from 16 *Eco*RI primers and 22 *Mse*I primers were employed in this stud (Table 1).

Table 1. Screening of AFLP markers associated with the resistance to *M. exigua*

Screening Material (a)	No. of primer combinations	No. of polymorphic AFLP bands	No. of AFLP markers associated with the resistance to M. exigua (b)
Timor hybrid-derived genotypes	232	403	10
F_2 individuals	110	161	5
Total	342	564	15

[a] *Screening material included either 2 resistant (T5296 and T17925) versus 2 susceptible (T17928 and T18137) Timor hybrid-derived genotypes or 2 resistant versus 2 susceptible F_2 individuals from a cross between Et6 (susceptible parent) and T5296 (resistant parent)*
[b] *AFLP makers appearing associated with the resistance to M. exigua in a set of 10 F_2 individuals (5 resistant and 5 susceptible) from a cross between Et6 (susceptible parent) and T5296 (resistant parent)*

More than 564 markers of "canephora" chromosome segments introgressed in *C. arabica* lines were generated. Screening material included either 2 resistant (T5296 and T17925) versus 2 susceptible (T17928 and T18137) Timor hybrid-derived genotypes or 2 resistant versus 2 susceptible F_2 individuals from a cross between Et6 (susceptible parent) and T5296 (resistant parent). A total of 15 AFLP makers exhibiting a strict association with the resistance to *M. exigua* in a set of 10 F_2 individuals (5 resistant and 5 susceptible) from a cross between Et6 (susceptible parent) and T5296 (resistant parent), were identified (Figure 1). Further linkage analysis was performed in the F_2 (Et6 x T5296) population and a localised genetic map of the chromosome segment carrying the *M. exigua* resistance gene was established. The low rate of recombination indicated these markers could be useful landmarks for map-based cloning of the resistance gene. With this purpose, a BAC library is being constructed.

COFFEE RUST RESISTANCE GENES

Introduction

The early recognition event between the plant R protein and the pathogen's avr gene product triggers the rapid activation of several plant defense responses that in concert lead to disease resistance. Among these responses is rapid localized cell death at the site of infection, termed the hypersensitive response (HR), which is thought to limit pathogen invasion in plants. In the specific interaction between coffee and the rust fungus *H. vastatrix*, the leaf resistance reaction appears as chlorotic flecks usually associated with punctiform tumefactions, visible 12-15 days after inoculation (Rodrigues et al., 1975). Microscopic examination of coffee leaves during a time-course of infection showed that cellular death events are commonly observed at the infection site (guard cells) by three days post-inoculation (Silva et al., in press).

The aim of our study is to characterize genes early expressed during the hypersensitive reaction of coffee (*C. arabica*) to the rust fungi. We initiated an EST (Expressed Sequence Tag) project to establish a reference catalogue of genes that are differentially expressed during

the resistance reaction. ESTs are short DNA sequences read from randomly chosen cDNA clones. In our approach, sequencing reactions are performed on selected cDNA clones obtained from a subtractive cDNA library, and the resultant ESTs are compared with sequence databases for identification (Figure 2). Cloning and functional analysis of genes involved in the *H. vastatrix* resistance pathways will not only lead to identification of some genetic components controlling disease resistance and cell death, but also allow to manipulate these non-pathogen specific genes to achieve broad spectrum resistance in coffee.

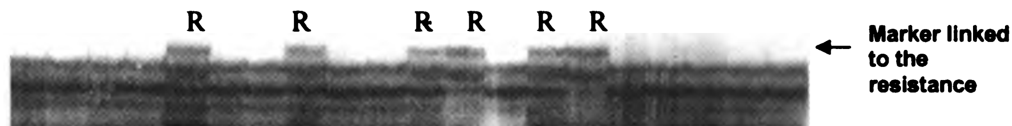

Figure 1. Example of AFLP Marker linked to the resistance to *M. exigua* derived from the Timor hybrid. Plants showing resistance to *M. exigua* are indicated by R (Resistance)

Material and methods

Plant variety, fungal strains, and inoculation

Coffea arabica accession 110/5 was inoculated with *H. vastatrix* isolates, either race II (isolate 1427) eliciting an RH (incompatible interaction) or with race XIV (isolate 178a) giving rise to rust disease (compatible interaction), using standard conditions (D'Oliveira and Rodrigues, 1961). Control plants were inoculated with water. Coffee leaves collected at various times after inoculation were immediately frozen in liquid nitrogen and stored at – 80°C until RNA extraction.

RNA extraction

Total RNAs were extracted from coffee leaves using the RNeasy Plant kit (Qiagen, France) completed by a DNAse treatment. Quality and concentration of RNA were checked on denaturing agarose gel and by absorbance measurements at 230, 260 and 280 nm on a UV spectrophotometer.

Construction of the subtractive cDNA library

Based on cytochemical observations (Silva et al., 1999, and in press) which showed that 48 hours after inoculation 17.5% of infection sites exhibited dead cells, we choosed the times 24 and 48 hours to construct a coffee cDNA library enriched in sequences specifically expressed during the HR. Total RNAs were extracted from coffee leaves undergoing incompatible or compatible interactions at 24 h and 48 h after inoculation. Total RNAs extracted at 24 h and 48 h after inoculation were pooled (500 ng each) to obtain 1µg total RNA of resistant and susceptible samples. cDNAs were synthesized using the SMART-PCR cDNA synthesis kit (Clontech, USA). The subtractive cDNA library was obtained using the suppression subtractive hybridization (SSH) method (Diatchenko et al., 1996) developped in the PCR-Select cDNA Subtraction kit (Clontech). The tester (resistant) cDNA sample was subtracted twice by the driver (susceptible) cDNAsample following the manufacturer's recommendations. Subtracted cDNA sequences were further ligated into a plasmid vector and

used to transform *Escherischia coli* competent cells (pGEMt-easy kit, Promega, France and TOPO cloning kit, InVitrogen, France).

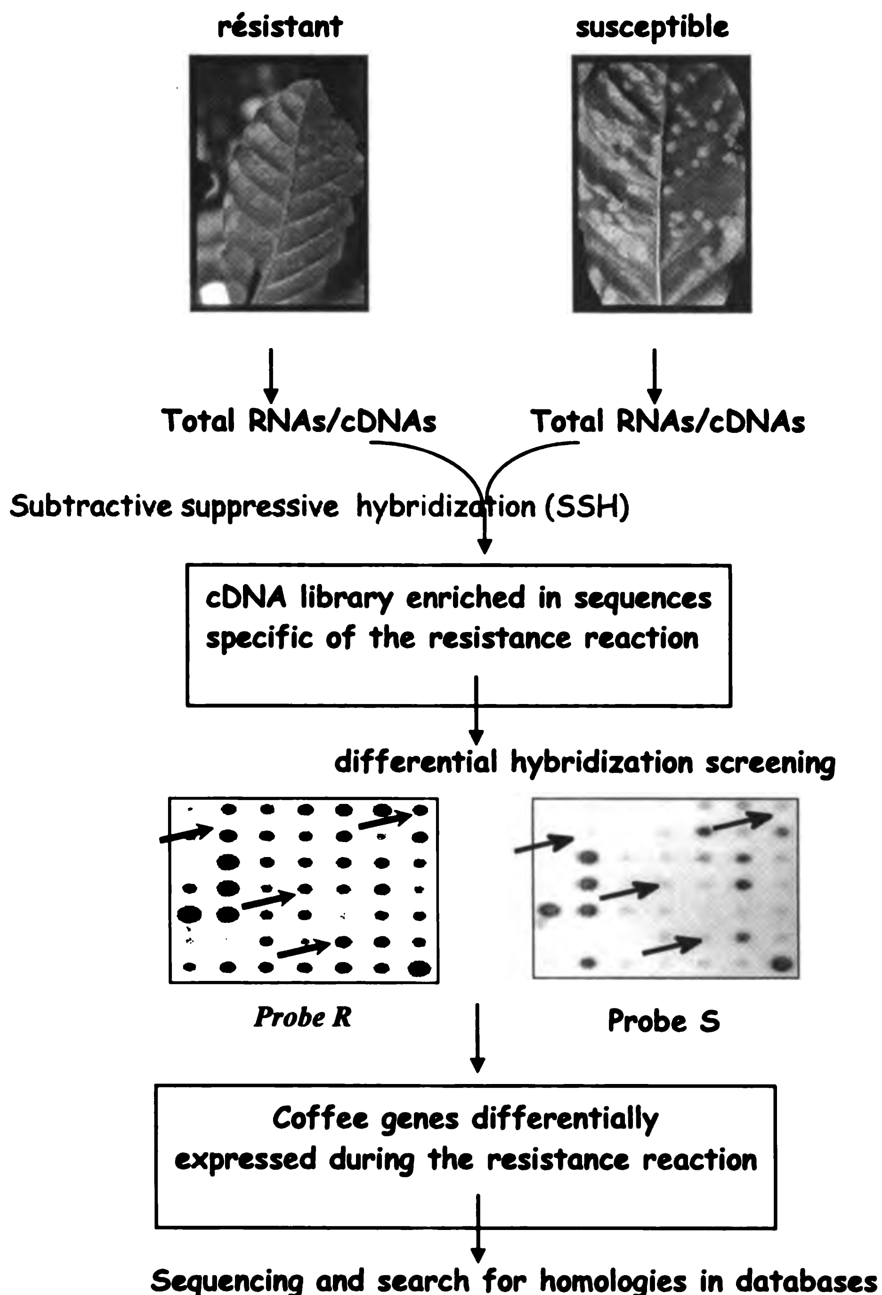

Figure 2. Scheme showing the strategy developped to isolate genes early expressed in the resistance reaction of coffee to rust disease. Arrows indicate some examples of clones that were selected based on the intense hybridization signal with probe R and the weak signal obtained with probe S

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Differential screening of subtracted cDNA clones

Selection of cDNA clones specifically expressed in the resistant samples was improved by differential screening of the cDNA clones presents in the subtractive library. After purification, recombinant bacterial colonies were arrayed on Nylon membranes (Hybond NX, Amersham, France) and hybridized with the tester (R) and driver (S) radioactively-labelled probes as indicated in the PCR-Select Differential Screening kit User Manual (Clontech). Clones showing an intense hybridization signal with probe R and a weak signal with probe S were selected. Confirmation of screening was made by a secound round of differential hybridization on purified plasmids extracted from selected clones (50 and 100 ng) arrayed on Nylon membranes (Hybond N+, Amersham).

Sequencing and bioinformatic analysis of cDNA clones

Selected cDNA clones were sequenced (Centre de séquençage de l'INRA Nancy, France) and homologies with sequences present in international databases were searched using algorithms developped at the National Center for Biotechnology Information (NCBI) web site (www.ncbi.org).

RESULTS AND DISCUSSION

Using the suppression subtractive hybridization method (Diatchenko et al., 1996), a coffee cDNA library enriched in sequences specifically expressed during the HR was obtained. In addition, recombinant clones were screened by differential hybridization against complex cDNA probes generated from resistant and susceptible mRNA pools (Figure 2). Thirty cDNA clones selected based on their hybridization signals were sequenced. Most of them (80%) showed significant homologies with plant sequences in databases. More than a third of the sequenced clones showed high similarities (P value $>10^{-20}$) with proteins which role in plant defence reactions have been suggested or demonstrated, such as chitinases, heat shock proteins, cytochrome P450, metallothioneins and ionic channels (Choi et al., 1996; Clough et al., 2000; Guy and Li, 1998; Van Loon and Van Strien, 1999; Whitbred and Schuler, 2000). Other clones either belonged to the category of house-keeping genes or had homologies with putative proteins for which no function had been assessed. These latest cDNA sequences might be coffee-specific.

Future work will aim at understanding the role of selected clones and sequences showing no similarities with known proteins in the mechanism of coffee resistance to parasites. Genes isolated in coffee plants will be studied for their *in vivo* function and the transcriptional regulation of their expression.

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