Transcriptome comparison of resistant and susceptible *Hevea brasiliensis* cultivars infected by *Microcyclus ulei*

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ABSTRACT

- *Background and Aims* South American Leaf Blight (SALB) is caused by an ascomycota, *Microcyclus ulei* (P. Henn.) v. Arx., and is the most damaging disease in Latin America and the most important threat for rubber tree plantations in Asia and Africa which produce 95% of the world rubber with susceptible genotypes. Because few information are available to understand the biological process of the disease development, suppression subtractive hybridization (SSH) approach was developed in order to identify genes differentially expressed at different stages of the pathogenic interaction with *Microcyclus ulei*, with a particular interest for defence and stress-related genes.
- *Methods* To identify candidate genes and gain better understanding of the host-pathogen interaction in the pathosystem *Hevea brasiliensis – Microcyclus ulei*, Suppression Subtractive Hybridization libraries were compared. Leaflets of the MDF 180 partially resistant cultivar and the PB314 susceptible cultivar were inoculated with *M. ulei*. Using 6 to 72 h.p.i, 4 to 28 d.p.i, and 34 to 58 d.p.i infected and healthy leaflets, five cDNA libraries, highly enriched for *M. ulei* -induced genes were prepared.
- *Key Results* Within 8027 clones randomly picked and sequenced from the libraries, 1165 singlets and 458 contigs for a total of 1623 non-redundant sequences were obtained. The redundancy of each SSH libraries were variable, ranging from 87% in PB314 4-28 d.p.i libary to 23% in MDF180 4-28 d.p.i library. Sequence analysis allowed the assignment of a putative functional category for 38% of sequences, whereas 15% of sequences corresponded to unknown function and 47% did not show any significant similarity with other proteins present in the databank. Clustering of the whole sequence reveal a high degree of dissimilarity between the genes isolated in each library. Moreover, the obtained ESTs compared with the *Hevea* latex transcriptome, appear highly specific of the leaf tissue. The comparison of the five libraries displayed a drastic diminution of the number of over-expressed genes in PB314 genotype during the infection process, differently from MDF180 genotype for which the number of

genes related to stress and defence display an increase of 56%. These preliminary results are put together with symptomatic and histological observations.

Introduction

South American leaf blight cause by the ascomycota, *Microcyclus ulei* (P. Henn.) v. Arx., is the most damaging disease in Latin America and the most important threat for rubber tree plantations in Asia and Africa which produce 95% of the world rubber with susceptible genotypes. The fungus attacks mainly the young leaflets but also young stems and immature fruits, and induces repeated defoliations, die-back of the canopy and even the death of the trees. Recently, Lieberei (2007) reviewing the physiological and biochemical features in the *Hevea* - *Microcyclus ulei* interaction gives five developmental levels which take place from the germination of the conidiospores to the liberation of mature ascospores in this host-pathogen interaction:
(1) Germination and pre-penetration phase with early impact of the plant on the conidiospores' infection potential from 0 to 12 hours post inoculation (hpi).
(2) Penetration and initial infection phase with induction of early resistance responses from 6 to 24 hpi.
(3) Leaf colonization, intercellular growth and ramification of hyphae from 24 to 96 hpi.
(4) Formation of conidiophores, which leads to differences in the spore number and spore distribution in and around the lesion from 4-5 days post inoculation (dpi) to 28 dpi with a maximum 12 dpi.
(5) Perithece formation and liberation of mature ascospores from 10 days to 540 dpi.

Intensity and time occurrence of each level enable a quantification and a description of the resistance or susceptibility (Junqueira et al., 1990, Rivano, 1992). Recently, the original resistance of the MDF180 cultivar has been reported in industrial plantation scale in two Brazilian areas (Bahia and Mato Grosso) and in French Guiana (Le Guen et al., 2008). In Brazil, MDF 180 resistance performance appears similar : moderate sporulation, no perithece, and no observable defoliation of the affected trees. In French Guiana, no sporulation and no perithece were observed on this clone. This resistance behaviour was confirmed in controlled conditions with strains from Bahia and French Guiana.

In order to dissect the molecular changes during the SALB resistance or susceptibility process in a comprehensive manner at the genomic level, and to identify genes involved in this process, it is necessary to survey global gene expression pattern following the infection by *M. ulei*. To obtain information about the differences between the mRNA expression profiles on resistant and susceptible cultivar, we used the suppression subtractive hybridization (SSH) approach in order to both normalize libraries (and thus minimize clone redundancy) and achieves a greater than 1000-fold enrichment of differentially expressed cDNAs (and thereby isolate weakly differentially expressed genes) (Diatchenko *et al*., 1999). This method was successful in identifying various differentially expressed genes in plant-pathogen interactions such as *Venturia inaequalis* and apple (Degenhardt *et al*., 2005), cacao x *Moniliophthora perniciosa* (Gesteira *et al*., 2007), *Hemileia vastatrix* and coffee (Fernandez *et al*., 2004), *Verticillium dahliae* and cotton (Zuo *et al*., 2001) or in symbiotic interactions such as *Pisolithus tinctorius* and

Eucalyptus (Voiblet *et al*., 2001), *Sinorhizobium meliloti* and *Medicago truncula*ta (Godiard *et al*., 2007). In rubber tree, it was recently used to identify in the latex novel gene markers of tapping panel dryness (TPD) (Venkatachalam *et al*., 2007).

Here, the generation and analysis of the first ESTs from *Hevea – M. ulei* interaction are reported. The main goal of this work was to explore the transcript profiles of infected leaves from the SALB resistant cultivar MDF180 and to compare it to a susceptible cultivar, PB314. Five SSH libraries were prepared. The first one (MDF180 6-72hpi) and the second one (PB314 6-72hpi), contain cDNA fragments corresponding to genes over-expressed respectively in MDF180 and PB314 infected leaves, 6 to 72 hpi. The third one (MDF180 4-28dpi) and the fourth one (PB314 4-28dpi) contain cDNA fragments corresponding to genes over-expressed respectively in MDF180 and PB314 infected leaves, 4 to 28 dpi. The fifth one (PB314 34-58 dpi) correspond to genes over-expressed in infected leaves 34 to 58 dpi. As a first report of global expression of *Hevea* leaf genes, a comparison with hevea latex genes rich in defence and stress proteins were achieved.

Materials and methods

Plant variety, fungal strains, and inoculation

Hevea brasiliensis cv. MDF180 and cv. PB314 were clonally propagated by grafting on rootstocks and kept in a greenhouse in the Michelin Plantation (Ituberá, Bahia). Plants which a first mature flush were transferred from the greenhouse to the inoculation chamber, enabling a rigorous control of environmental parameters: temperature of 24 °C ± 2 °C, relative moisture superior to 95% and a 12:12 photoperiod. Conidia from three strains (FTP13, FTP19, FTP22) coming from lesions obtained by inoculation of a susceptible cultivar were suspended separately in sterile water with 0.05% Tween 80, and the final concentration was adjusted to 2.10^5 mL^{-1} conidia (Mattos, 2003). The lower surface of different young leave (stage B2) was inoculated with the conidia suspensions of each strain, using a Paasche airbrush set. Sterile water with 0.05% Tween 80 was also sprayed on leaves of separate plants as controls. For each inoculation, infected plants of the two cultivars were observed during at least 12 days pi. to verify the efficiency of the infection.

Leaves were collected 6, 12, 24, 48, 72, 96, 240 hpi and then every 6 days to 58 dpi corresponding at different development stage (Fig. 1), quickly dissected to remove the central vein, then immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction

For each sample, three leaves, each one infected with different strain, were mixed and total RNAs were extracted using the Concert™ Plant RNA Reagent (Invitrogen Corporation, Carlsbad USA). For MDF180 6-72hpi and PB314 6-72hpi SSH libraries, 1 µg of total RNA was obtained from the mix of 200 ng of RNA from infected leaves collected 6, 12, 24, 48 and 72 hpi. The same mixes were prepared for control leaves of the resistant and susceptible genotypes. For MDF180 and PB314 4-28 dpi libraries and PB314 34-58dpi libraries, the preparation of the mixes was identical as before according to the time pi (4, 10, 16, 22, 28 days pi and 34, 40, 46, 52, 58 days pi). This extracts were treated with DNAse in the presence of 40 units. of RNase out (Invitrogen). Quality and concentration of RNA were checked on 1% denaturing agarose gel.

Construction of the Hevea subtractive cDNA libraries

Total RNAs were used to produce SMART-cDNA using the SMART-PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The subtractive cDNA libraries kits were obtained using the PCR-Select cDNA Subtraction kit (Clontech). The tester (infected leaves) SMART-cDNA sample was subtracted by the driver (control) SMART-cDNA sample following the manufacturer's recommendations (forward subtraction).

All PCR amplifications were carried out using the Advantage cDNA PCR kit (Clontech). Subtracted cDNA sequences were cloned into a pGEM-T (Promega, France) and used to transform by

electroporation *Escherischia coli* electro-competent cells (ElectroMAX™ DH10 cells, Invitrogen). White colonies were randomly picked and cultured in 1 mL of LB-Amp medium in 96-well plates at 37 °C.

cDNA Sequencing

DNA sequencing was performed at the genomic laboratory of the State University of Santa Cruz (Bahia, Brazil). Plasmid DNA from cDNA clones was extracted and sequencing reactions were performed with for a 5µL reaction using, 2µL DYEnamic™ ET terminator sequencing premix, 0.3µL M13(-26) reverse primers (3.2 pmole) and 2.7µL plasmid DNA. Cycle sequencing was performed over 40 cycles (95 °C for 10s; 50 °C for 15s; 60 °C for 1.33 min) in a Eppendorf thermocycler. Samples in 96-well plates were loaded on to a MegaBACE™ 1000 capillary sequencer (Amersham Biosciences).

Sequence analysis

Raw sequence chromatograms trace files were processed by the program PHRED quality ≥10 (allowing 1% nucleotide with Phred quality <10) using a Perl script (Ewing et al., 1998). The plasmid vector sequences was removed with cross-match, the poly(A) or poly (T) tail was removed. In an ultimate step, sequence end in 5' and 3'ends were manually removed using the *RSAI* restriction site position. Finally, the redundancy was eliminated by contig assembling with CAP3 for each library datasets and also for the whole library dataset (Huang and Madan, 1999). After this trimming process, only unique sequences and contig longer than 90bp were included in the dataset. For putative function determination and annotation sequences were compared with the public sequence database (http://www.ncbi.nih.gov/BLAST/) using BLASTX and TBLASTX. Alignments showing similarity with an expected value ≤ 1.10^{-4} were considered significant. Additional information about the putative function of the ESTs and classification in functional group were obtained using Pfam and Blast2GO programs (Conesa and Götz, 2008). To compare with *Hevea* latex transcripts, the EST clusters were also compared with 3.347 sequences from *Hevea* available in the Genbank database using BLASTX and TBLASTX.

Histological observations of leaflets during infection

Leaflets from inoculated and non-inoculated MDF180 and PB314 grafted plants were harvested at 7 and 10 days after inoculation (dai) and immediately fixed for at least 5 h in 0.5 M phosphate buffer pH 7.2, containing 2% paraformaldehyde, 1% glutaraldehyde. Plant tissues were then dehydrated in an alcohol series. Samples were subsequently embedded in Histo Resin (Leica, Wetzlar, Germany). Four mm-thick sections were double stained with naphthol blue-black and Periodic Acid-Schiff (PAS), which were observed in a CX41 microscope (Olympus, Denmark) under white light, and photographed with a CAMEDIA C-7070 camera (Olympus, Denmark).

Results

Five SSH libraries enriched for sequences differentially expressed during the *Hevea* and *M. ulei* interaction were generated from MDF180 and PB314 cultivars. In order to maximize the detection of over-expressed transcripts in infected leaf in relation to healthy leaf but present in a few copies principally during the early stage of infection where a few cells are in contact with the fungus, we decided to separate early interaction processes (from the germination of the spore to the intercellular spread of the fungus) to latter interaction processes (from conidiophore to perithece formation and maturation). Moreover, using the Peruvian wild cultivar, MDF180, and a back-crossed cultivar PB314, we suppose that the basal expression of the genes could be very different between the two cultivars and could result in the isolation of several false positives ESTs in a "resistant" *vs*. "susceptible" hybridization. Consequently, we preferred to use the SSH in the forward direction, "infected" *vs*. "healthy", followed by an *in silico* comparison of the libraries.

A total of 8027 clones were generated and after trimming for low quality, vector contamination and shortness (<90bp), a total of 1890 and 2382 sequences, were obtained respectively for MDF180 and PB314 (Table 1). The useful sequences varied from 33% to

77% percent depending of the library. The length of good quality sequences had an average size of 346 bp.

Global analysis of Hevea – M. ulei genes

For the 6 to 72 hpi libraries, the initial data set of 1081 and 1076 sequences from MDF180 and PB314 were reduced to 352 and 751 consensus sequences (unigenes). The MDF180 unigen set comprised 146 contigs (875 sequences) and 206 singletons, whereas the PB314 unigen set comprise 125 contigs (450 sequences) and 626 singletons (Table 1). The number of ESTs forming each contig varied between two (50 cases) and 44 (one case) for MDF180 (Fig. 2-B) and two (76 cases) and 51 (one case) for PB314 (Fig.2-A). The values of redundancy was estimated at 67% and 30% for MDF180 and PB314 (Table 1) indicating for PB314 library a possibility to reveal new sequences generating and analysing more sequences.

For the 4 to 28 dpi libraries, the 809 and 715 sequenced clones were clustered into 111 contigs (301 sequences) for MDF180 and 58 contigs (676 sequences) for PB314, leaving respectively 619 and 97 singletons (Table 1). The number of ESTs forming each contig varied between two (76 cases) and 12 (one case) for MDF180 (Fig. 2-B), and between two (11 cases) and 85 (one case) for PB314 (Fig.2-A). The redundancy was low for MDF180 (23%) and high for PB314 (87%). High redundancy (94%) was also obtained in the PB314 4-28dpi library (Table 1). Distribution of the contigs on the number of ESTs sequences strengthen this observation indicating a large number of contigs constituted of few ESTs in the PB314 6-72 hpi library and reduced number of contigs constituted of few sequences in benefit of contigs constitutes with a large number of ESTs in PB314 4-28 dpi. This evolution was more accentuated, in PB314 34-58 dpi presenting contigs constituted with up to 200 sequences (Fig. 2-A). This result indicated a reduction in the number of over-expressed genes in the susceptible cultivar from 4 days to 58 dpi comparing with MDF180 that maintained a large over-expressed set of genes all long the infection process (Fig.2-B).

Clustering analysis of the 4272 sequences result in 1165 singletons and 458 contigs (Table 1), permits to build the Venn diagrams indicating the number of sequences specific to the cultivar and/or infection time (Fig. 3). Independently of the infection time, 767 sequences were only identified in MDF180 infected leaves and 709 sequences in PB314 infected leaves. The number of sequences over-expressed in the two cultivars was 147 (Fig. 3-A). Considering the time of infection, 99.1% of the PB314 over-expressed genes were detected in the first step of infection from 6 to 72 hpi, when only 39.8% of the MDF180 over-expressed genes were isolated in MDF180 cultivar (Fig. 3-B). From 4 to 28 days pi, only 83 over-sequences (11.7%) were identified specifically in PB314 cultivar when in the MDF180 cultivar, the number of over-expressed sequences (594 sequences) increased to 77.4% of the total number of specific sequences identified in this cultivar (Fig. C). Comparing the libraries of the PB314 cultivar (Fig. 3-D), few over-expressed sequences were common to two libraries and anyone was common to the three library. As indicated previously, the decrease in over-expressed genes was moreover considerable in the latter step of the infection : Only 18 over-expressed genes was detected in PB314 34-58 dpi library, when 727 and 69 specific genes were respectively detected in PB314 6-72 hpi and PB314 4-28 dpi library. The comparison between the two MDF 180 libraries

(Fig. 3-E), indicated also a high specificity of each library and only 64 common sequences (14.3% of the MDF180 unigen set).

Sequence annotation

Using manual annotation, expressed genes were classified into 10 functional categories according to the putative function of their homologous genes in the databases generated by BLAST analysis (Fig. 4).

For the 6-72 hpi libraries, putative functions were assigned to 356 and 753 sequences for MDF180 and PB314, leaving respectively 129 and 350 sequences without annotation and 57 and 121 matched ESTs with genes in GenBank database of unknown functions.

For the 4-28 dpi libraries, putative functions were assigned to 622 and 110 sequences for MDF180 and PB314, leaving respectively 296 and 70 sequences without matches and 86 and 13 matched ESTs with genes in GenBank database of unknown functions.

For PB314 34-58 dpi library, putative functions were assigned to 35 sequences, leaving 10 sequences without matches and 10 matched ESTs with genes in GenBank database of unknown functions.

In all the functional classes of the PB314 libraries were noted a drastic reduction of over-expression genes with the infection time when in MDF180 cultivar the number of sequences increased in quite all the functional classes (except signalling protein). Comparing MDF180 6-72 hpi and MDF180 4-28dpi library, the "stress response" functional class and the group of sequences with no similarity presented the higher increase (56%) of the number of over-expressed genes.

Comparison of the Hevea – M. ulei interaction unigene set with available Hevea latex sequences

The comparison of the *Hevea* - *Microcyclus ulei* interaction unigen sets with 3.347 GenBank non-redundant sequences from *Hevea* latex (Table 3), display that an average of 80% of the isolated genes are specifically expressed in *Hevea* leaves. Transcriptomic analysis of latex (Ko et al., 2003), reveals that among seven functional groups accounting for more than 51% of transcripts, the second most abundant transcripts in latex were defence or stress-related genes. This confirms that defence is presumably one important function of the laticifers. Some of this transcripts were also isolated in leaf (Table 3) as some chitinases (hevamine, acidic chitinase), protease (beta-VPE, putative preprocysteine proteinase), protease inhibitor (cystatin, cysteine protease inhibitor, protease inhibitor protein 1), pectine esterase inhibitor, ROS-detoxifying protein (thioredoxin h, thioredoxin-dependent peroxidase, ascorbate peroxidase, MnSOD, catalase), programmed cell death inhibitor (Bax inhibitor) and other stress proteins (Class I heat shock protein, heat shock protein 90, symbiosis-related like protein, salt tolerance protein, alpha-hydroxynitrile lyase).

Discussion and conclusion

The results presented describe the first molecular resources of genes expressed in rubber tree leaves infected with a fungus. It was opportunely decided to build SSH libraries from 6 to 72 hpi leaves, 4 to 28 d.p.i leaves and 34 to 58 dpi to try to isolate genes expressed early, medium or late in the case of a partial resistance or susceptibility to South American Leaf Blight.

From 6 to 72 hours, according to Blasquez and Owen (1963), Hashim *et al.* (1978) and Garcia (1995), germination of the conidiospores and penetration of the fungus occur and the fungus spread in epidermal, sub-epidermal and parenchyma tissues in the partially resistant and susceptible cultivars. From 4 to 5 days, sporulation of the fungus is observed firstly on the lower surface of the leaflets and at 10 dpi, also in the upper surface of the leaflets in the most susceptible cultivar (Fig. 1-c and 1-h). In the case of strong attacks on young leaves, abscission can occur in susceptible clones preventing the formation of teleomorph (Fig 1-h). From 10 dpi, pycnidia form mostly on the upper surface around the lesion (Fig. 1-j) and with the maturation of the leaf, ascocarps form and become more massive (Fig 1-k) until one to three months after infection when occur the liberation of mature ascospores (Sambugaro *et al.*, 2003). Recently, the original SALB resistance of a Peruvian hevea genotype, MDF180 has been described (Le Guen *et al.*, 2008). From 160 *M. ulei* strains tested in controlled inoculated conditions, most of the symptoms of MDF180 cultivar were partial sporulating lesions (Fig. 1-c). The sexual phase of the fungus was never observed on this cultivar, either in controlled conditions or in natural infestation (Fig. 1-d and 1-e). Considering that MDF180 resistance holds for more than thirty years in areas very conducive to the *M. ulei*, it was defined this resistance as a durable resistance. Histological observations of 10 dpi leaflet of the MDF180 cultivar, revealed a delay of collapsed cell (Fig. 5-A) and a limitation of mycelium spread in this cultivar compared with a susceptible cultivar PB314), where the extensive spread of hyphae are associated to cell disruption (Fig 5-C). In MDF180, starch grains accumulated in chloroplasts (Fig. 5-B). An enlargement of the nucleus is observed principally in the lower dermal and epidermal cells, typical of an uncondensed chromatin (Fig. 5-D) indicating an intense transcriptional activity.

The results of the qualitative analyses of the transcriptome interaction presented in this study are consistent with this histological observations. During the first step of infection, a large number of genes are over-expressed in the resistant and susceptible cultivars. However, we can suppose that just a few of them are efficient to limit mycelium spread in the tissue like in the partially resistant cultivar and others can be inefficient or prejudicial to leaf tissue integrity like in the susceptible cultivar. Inside the functional group of genes related to stress and defence response, we detected specifically in the MDF180 6 to 72 hpi library, genes of phenylpropanoid biosynthesis like cinnamyl alcohol dehydrogenase, 4-coumarate CoA ligase involved in lignin biosynthesis pathway, MnSOD and catalase limiting ROS damaging, and pathogenesis related proteins as β-1,3-glucanase and proteinase-inhibitor. All this early up-regulated genes may contribute to limit fungal spread and tissue damaging. In the PB314 6 to 72 hpi library, 24 different genes are associated to stress responses, however up-regulation of a large part of them may be prejudicial to plant cell integrity as germin-like considered as a PRP with superoxide dismutase activity generating hydrogen peroxide that can be toxic to different types of attackers including plant cell tissues if not contained or detoxified by plant.

Peroxidase and thioredoxine peroxidase identified in this library can confer a protective role in cells through its peroxidase activity by reducing hydrogen peroxide. Among the PRP, chitinase, NtPRp27 like protein, PR10 (ribonuclease-like protein) were found and a proteinase-inhibitor 1, a serine protease yet identified in *Hevea* latex (Sritanyarat et al., 2006). Leucine Rich Repeat Protein has also been detected and different stress expressed proteins as symbiosis, salt tolerance, dehydration and heat shock proteins.

From 4 to 28 days p.i., in the MDF 180 library, several genes of the functional group “stress and defence” are in involved in cell structure protection as cystatin and serpin, Bax inhibitor1, defender against cell death, monodehydroascorbate reductase, catalase II and pectinesterase inhibitor. Transcripts coding for key enzymes of the lignin biosynthesis were also isolated as cinnamoyl-CoA-reductase and peroxidase which could be associated to lignin synthesis around the infection site as showed by Garcia *et al*. (1995b) and Sambugaro *et al*. (2004). PR proteins as PR1a, NtPRp 27 and hevamin were present in this library and may serve as essential function in plant life whether in defence or not (Van Loon *et al*., 2006). In the PB314 library, only 2 genes were identified, one R-gene (Cf5) and a stress response gene (metal tolerance protein), indicating an inefficiency of the plant to maintain or promote over-expression genes. In the PB314 - 28 to 54 days p.i. - library, only a papain-like cystein proteinase isolated also in the MDF180 6 to 72 hours p.i. was detected.

As far as is known this is the first EST resource from *Hevea* leaf and particularly from a *hevea* – parasite interaction. This new resource is important to better understand the molecular mechanism of resistance and susceptibility of *Hevea* to *Microcyclus ulei* and to develop functional molecular markers for marker-assisted selection.

References

Blasquez, C.H. and Owen, J.H. (1963) Histological studies of Dothidella ulei on susceptible and resistant clones. Phytopathology. 53, 58-65.

Conesa, A. and Götz, S. (2008) Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. International Journal of Plant Genomics. 1-12.

Degenhardt, J., Al-Masri, A.N., Kürkcüoglu, S., Szankowski, I., Gau, A.E. (2005) Characterization by suppression subtractive hybridization of transcripts that are differentially expressed in leaves of apple scab-resistant and susceptible cultivars of *Malus domestica*. Mol Gen Genomics. **273**: 326-335.

Diatchenko, L., Lukyanov, S., Chris Lau, Y.-F., Siebert, P.D. (1999) Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. Meth. Enzymol. **303**, 349-380.

Fernandez, D., Santos, P., Agostini, C., Bom, M.C., Petitot, A.S., Silva, M.C., Guerra-Guimarães, L., Ribeiro, A., Argout, X., Nicole, M. (2004) Coffee (*Coffea arabica* L.) genes early expressed during infection by the rust fungus (*Hemileia vastatrix*). Mol. Plant Pathol. 5, 527-536.

Garcia, D. (1995a) Contribution à l’étude de la résistance totale et partielle dans l’interation hôte x parasite *Hevea spp*. – *Microcyclus ulei*. Aspects histologiques et biochimiques des réactions impliquées dans ces résistances. Doctorate Thesis University of Montpellier II. France. 216 pp.

Garcia, D., Cazaux, E., Rivano, F., D’Auzac J. (1995b) Chemical and structural barriers to *Microcyclus ulei*, the agent of South American leaf blight, in *Hevea* spp. Eur. J. For. Path. **25**, 282-292.

Godiard, L., Niebel, A., Micheli, F., Gouzy, J., Ott, T. and Gamas, P. (2007) Identification of new potential regulators of the *Medicago truncatula-Sinorhizobium meliloti* symbiosis using a large-scale suppression subtractive hybridization approach. Mol. Plant Micr. Interaction. **20**, 321-332.

Hashim I. (1978) Histological and biochemical studies on South American leaf blight of *Hevea* species. Doctorate Thesis, Trinidade, West Indies. 237 pp.

Huang, X. and Madan, A. (1999) Cap3: a DNA sequence assembly program. Genome research **9**: 868-877.

Junqueira, N.T.V., Lieberei, R., Kalil Filho, A.N. and Lima M.I.P.M. (1990) Components of partial resistance in *Hevea* clones to rubber tree leaf blight, caused by *M. ulei*. Fitopatologia Brasileira. 15: 211-214.

Ko, J. H., Chow, K. S. and Han, K. H. (2003) Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree). Plant Mol Biol. **53**: 479-92

Leal, G.A., Albuquerque, P.S., Figueira, A. (2007) Genes differentially expressed in *Theobroma cacao* associated with resistance to witches'broom disease caused by *Crinipellis perniciosa*. Mol. Plant Pathol. **8**, 279-292.

Van Loon, L.C., Rep, M. and Pieterse C.M.J. (2006) Significance of inducible defense-related proteins in infected plants. Ann. Rev. Phytopathol. 44, 135-162.

Le Guen, V., Guyot, J., Mattos, C.R.R., Seguin, M., Garcia, D. (2008) Long lasting rubber tree resistance to *Microcyclus ulei* characterized by reduced conidial emission and absence of teleomorph. Crop Protection. **27**, 1498-1503

Mattos, C.R.R., Garcia, D., Pinard, F., Le Guen, V. (2003) Variablidade de isolados de *Microcyclus ulei* no Sudeste da Bahia. Fitopatologia brasileira. 28, 502-507.

Venkatachalam, P., Thulaseedharan, A. and Raghothama K. (2007) Identfication of expression profiles of tapping panel dryness (TPD) associated genes from the latex of rubber tree (*Hevea brasiliensis* Muell. Arg.). Planta. Published online.

Voiblet, C., Duplessis S., Encelot, N., Martin, F. (2001) identification of symbiosis-regulated genes in *Eucalyptus globulus-Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. The Plant Journal. 25, 181-191.

Rivano (1992) La maladie Sud-Américaine des feuilles de l'hévéa. Etude en conditions naturelles et contrôlées des composants de la résistance partielle à *Microcyclus ulei* (P. Henn.) V. Arx. Thèse de doctorat, University of Orsay, France. 232pp.

Sambugaro, R., Furtado, E.L., Rodella, R.A., Mattos, C.R.R. (2003) Anatomia foliar de seringeuira (*Hevea* spp.) e desenvolvimento da infecção por *Microcyclus ulei*. Summa Phytopathologica.

Sritanyarat, W., Pearce, G., Siems, W.F., Ryan, C.A., Wititsuwannakul, R., Wititsunwannakul, D. (2006) Isolation and characterization of isoinhibitors of the protease inhibitor I family form the latex of the rubber trees, *Hevea brasiliensis*. Phytochemistry. 67, 1644-1650.

Zuo, K., Wang, J., Wu, W., Chai, Y., Sun, X., Tang, K. (2005) Identification and charaterization of differentially expressed ESTs of *Gossypium barbadense* infected by *Verticillium dahliae* with suppression subtractive hybridization. Molecular Biology. 39, 191-199.

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Figure 1: Symptoms of SALB in MDF180 and PB314. Three days after infection, no symptoms are observed in leaves (a, f). Seven days after infection chlorotic spots appear on upper surface but sporulation of the fungus are not visible in MDF180 (b) cultivar, whereas in PB314, grey-black lesions (g) appear associated with lamina distortion. Ten days after infection, weak sporulation could be observed in the lower surface in MDF180 cultivar (c) and in PB314, sporulation can be noted in the two leaflet faces (h) associated to the radial and translaminar spread of the fungus. When the fungus attack is severe, the leaf usually abscisses. Older leaves with the same symptoms do not generally fall. As the leaf hardens the lesions lose their powdery appearance and become brownish (d and i). Fifty eight days after infection, any stromata was observed in the upper surface in MDF180 cultivar (e) whereas in PB314 cultivars, black teleomorph form (j). They increase in number and size, forming clusters and ring-like groups (k).

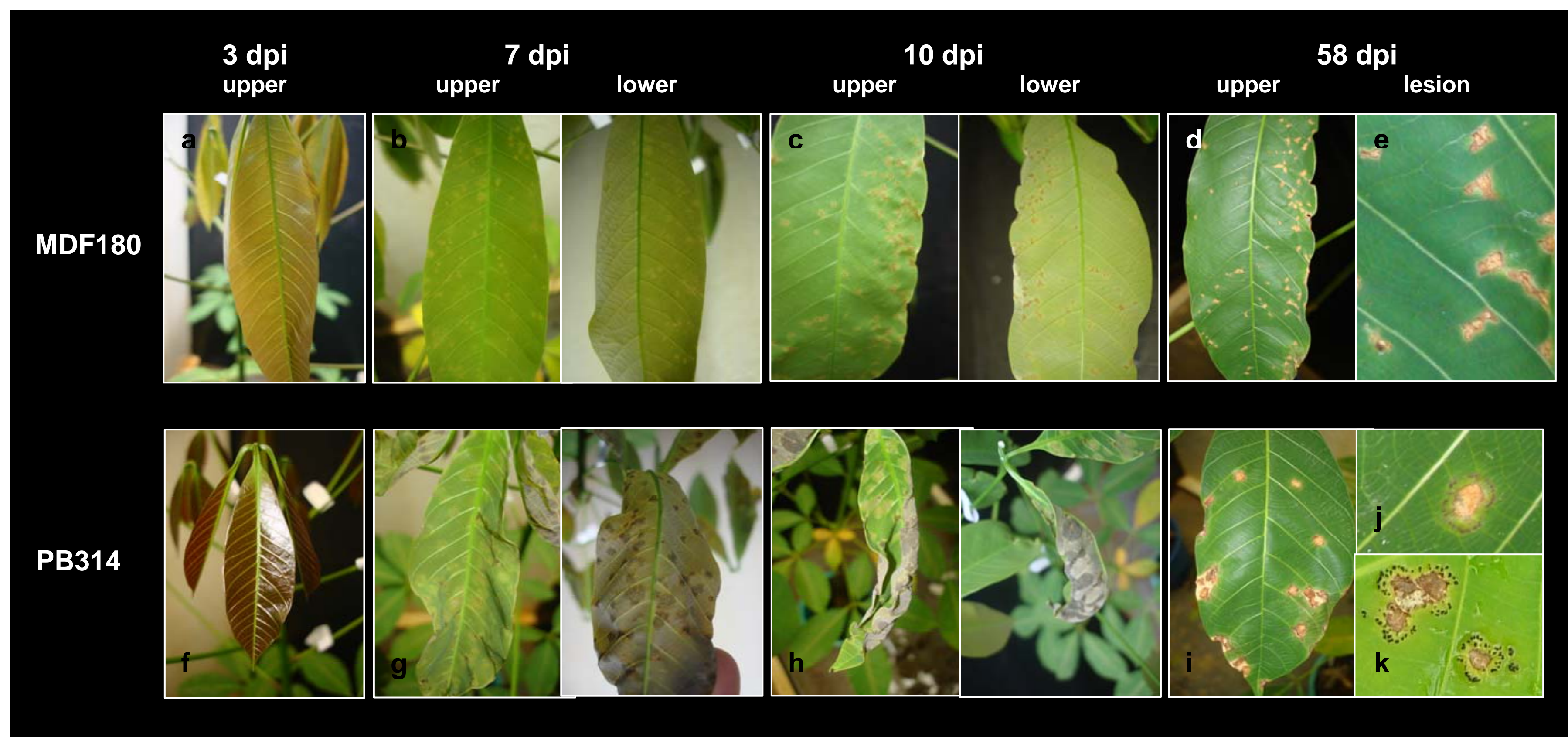


Table 1. Summary of the cDNA libraries MDF180 and PB314 created in this study with 6 hours to 58 hours post-infected leaves.

Library	No. of sequences generated	No. of sequences analysed[1]	Singleton (%)[2]	Contigs[3]	Unigene size (%)[4]	Mean size of the sequences (bp)[5]	Redundancy (%)[6]
MDF180 – 6 to 72 hpi	1776	1081 (61)	206 (19)	146	352 (33)	401	67%
MDF180 – 4 to 28 dpi	1790	809 (45)	508 (63)	111	619 (77)	302	23%
PB314 – 6 to 72 hpi	1849	1076 (58)	626 (58)	125	751 (70)	339	30%
PB314 – 4 to 28 dpi	884	715 (80)	39 (5)	58	97 (14)	352	87%
PB314 – 34 to 58 dpi	1728	591 (34)	11 (2)	24	35 (6)	437	94%
Contigated sequences	8027	4272	1165	458	1623	346	62%

[1] Vector sequences and sequences of low quality or smaller than 90 bp were eliminated.
[2] The singletons presents in each library independent of other libraries. The percentage was calculated as number of singletons/number of sequences analysed from library.
[3] The contigs present in each library independent of other libraries.
[4] The unigene set of each library is the sum of singleton plus contigs for the library.
[5] The mean size of the unigene sequences.
[6] The redundancy of each library calculated as 1 - (unigen library/number of sequence analysed)

Figure 2 : Histogram showing the distribution of ESTs by contigs. The contig size is the number of EST/contig. (A) SSH libraries from PB314; (B) SSH libraries from MDF180.

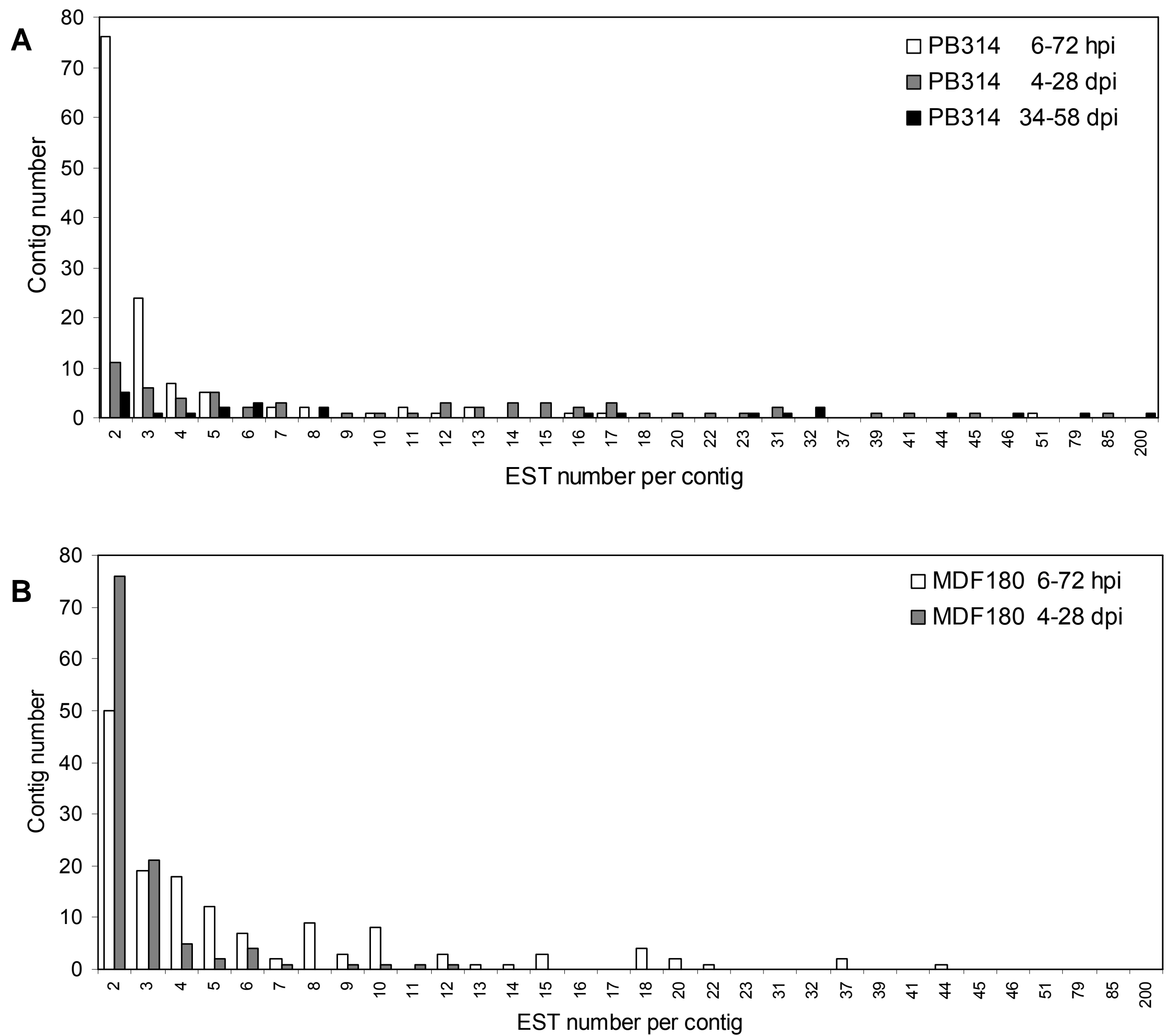


Figure 3 : Venn diagrams of the distribution of the contigated sequences present in SSH *Hevea* libraries.

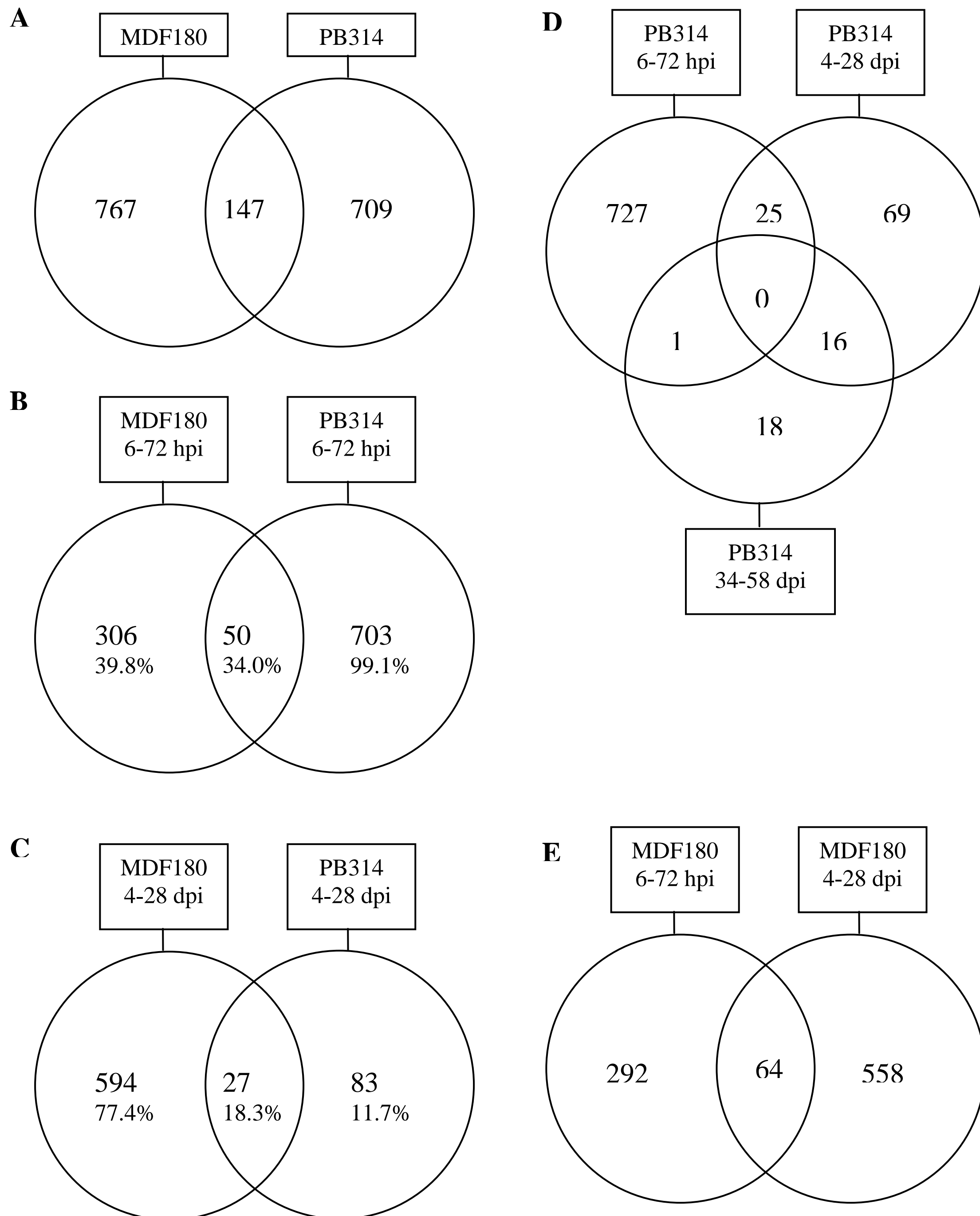
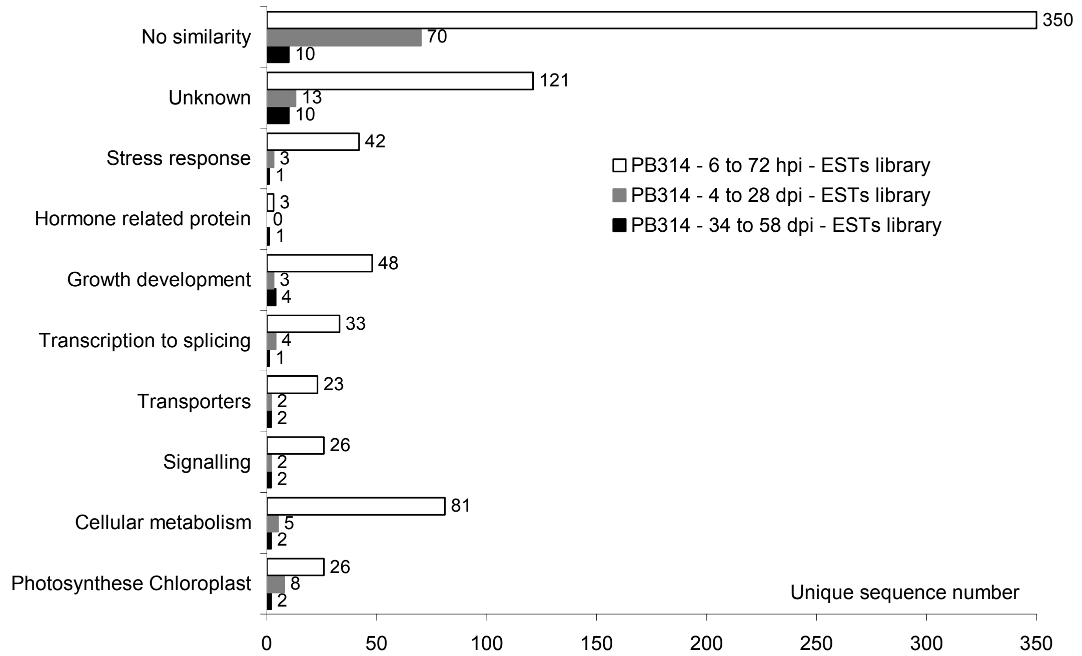


Figure 4: Distribution into functional classes of the unigene sequences identified in the *Hevea – Microcyclus ulei* interaction SSH libraries. Only one class was assigned to each sequence.



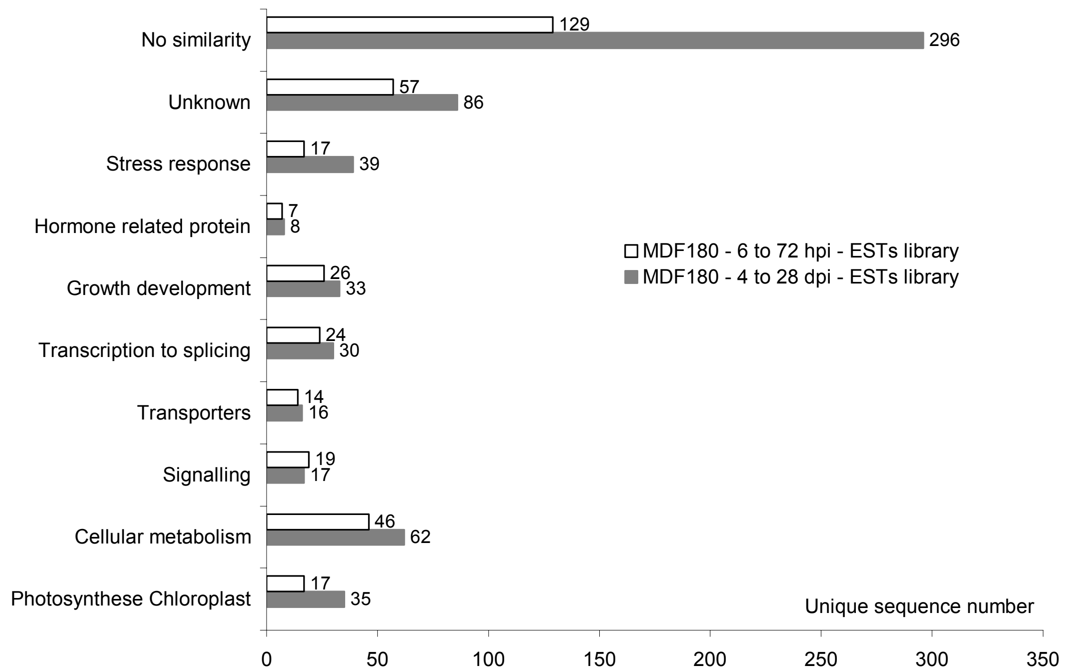


Table 3: Comparison of the *Hevea* - *Microcyclus ulei* interaction unigen sets with the 3.347 Genbank unique sequences from *Hevea* latex.

Library	No. of leaf specific sequences (%)	No. of similar sequences to latex
MDF180 6-72 hpi	273 (77.5)	79
PB314 6-72 hpi	584 (77.7)	167
MDF180 4-28 dpi	487 (78.6)	132
PB314 4-28 dpi	85 (87.6)	12

Table 4 : Sequences identified in MDF180 and PB314 libraries related to R-gene, stress and defence proteins.

Accession n°	BLAST sequence homology [*Species*]	*E*-value	sequence size	Library[†]
R-gene				
gb|AAT28308.1	leucine-rich repeat receptor-like protein kinase [*Pyrus pyrifolia*]	4E-68	429	3
emb|CAE76632.1	leucine rich repeat protein [*Cicer arietinum*]	3E-37	283	3
dbj|BAD94317.1	Cf-5 disease resistance protein - like [*Arabidopsis thaliana*]	4E-07	192	4
Chitinase				
gb|AAQ07267.1	acidic chitinase [*Ficus awkeotsang*]*	3E-33	428	1, 3
emb|CAC19408.1	hevamine [*Hevea brasiliensis*]*	9E-17	180	2
gb|AAK91891.1	putative elicitor inducible chitinase [*Solanum demissum*]	5E-34	397	3
Glucanase				
gb|AAN05325.1	Putative beta-1,3-glucanase [*Oryza sativa* (japonica cultivar-group)]	9E-24	403	1
gb|AAL30426.1	beta-1,3-glucanase [*Prunus persica*]	7E-35	413	1
Pathogeneses related protein				
gb|AAD33696.1	PR1a precursor [*Glycine max*]	3E-15	372	2
emb|CAF33484.1	putative pathogenesis-related protein [*Cucumis sativus*]	2E-12	175	2
emb|CAC16166.1	pathogenesis-related protein 10 [*Vitis vinifera*]	6E-35	710	3
gb|AAO22065.1	NtPRp27-like protein [*Solanum tuberosum*]	1E-26	391	3
gb|AAO22065.1	NtPRp27-like protein [*Solanum tuberosum*]	8E-26	297	1, 2
Protease				
ref|YP_063571.1	ftsH protease homolog [*Gracilaria tenuistipitata* var. liui]	2E-27	480	1
emb|CAE54306.1	putative papain-like cysteine proteinase [*Gossypium hirsutum*]	2E-36	555	1, 5
emb|CAE54306.1	putative papain-like cysteine proteinase [*Gossypium hirsutum*]	2E-19	415	2
emb|CAE54306.1	putative papain-like cysteine proteinase [*Gossypium hirsutum*]	3E-24	417	2
emb|CAH59426.1	cysteine protease 1 [*Plantago major*]	1E-59	521	2
ref|NP_567972.1	SLP2; subtilase [*Arabidopsis thaliana*]	2E-16	153	2
emb|CAJ02700.1	serine peptidase, clan SC, family S9A-like protein [*Leishmania major*]	8E-37	411	3
dbj|BAD94396.1	beta-VPE [*Arabidopsis thaliana*]*	2E-42	339	3
emb|CAB44983.1	putative preprocysteine proteinase [*Nicotiana tabacum*]*	1E-31	307	3
Protease inhibitor / pectinase inhibidor				
gb|AAP46156.1	protease inhibitor protein 1 *[Hevea brasiliensis*]*	6.E-27	443	3
ref|XP_469996.1	putative protease inhibitor [*Oryza sativa* (japonica cultivar-group)]	1E-27	437	1
emb|CAA11899.1	cystatin [*Castanea sativa*]*	2E-08	297	2
emb|CAH57572.1	cysteine protease inhibitor [*Populus tremula*]*	3E-48	564	2
emb|CAI43280.1	serpin [*Cucumis sativus*]*	2E-41	393	2
ref|NP_190322.1	pectinesterase/pectinesterase inhibitor [*Arabidopsis thaliana*]*	3E-05	99	2
ROS-detoxifying protein				
gb|AAO12854.1	thioredoxin h [*Pisum sativum*]*	3E-42	772	1, 3
emb|CAC36986.1	thioredoxin h [*Pisum sativum*]*	5E-38	383	3
emb|CAC84143.2	thioredoxin peroxidase [*Nicotiana tabacum*]*	8E-29	410	3

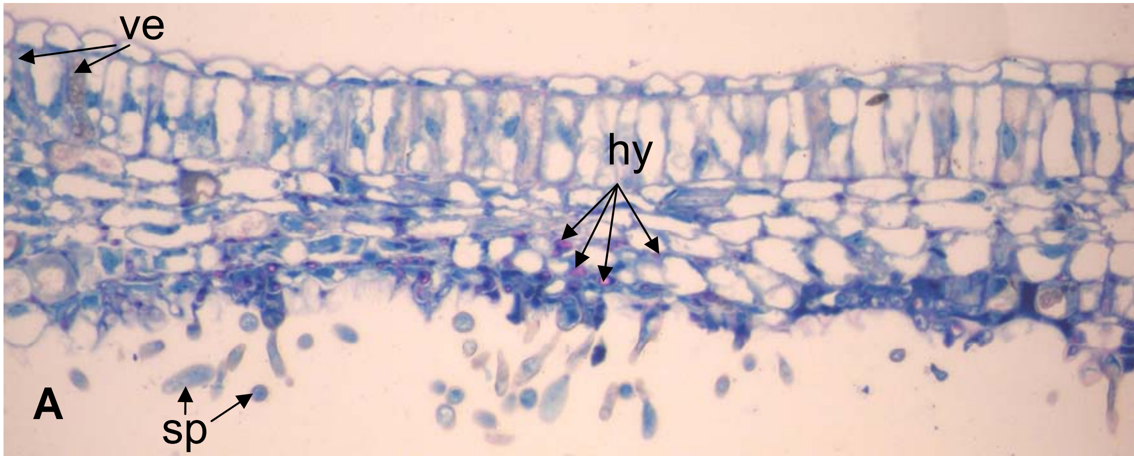
emb|CAH58634.1	thioredoxin-dependent peroxidase [*Plantago major*]*	4E-47	338	2
emb|CAA06996.1	ascorbate peroxidase [*Hordeum vulgare* subsp. *vulgare*]*	2E-12	416	2, 3
gb|AAL35365.1	ascorbate peroxidase [*Capsicum annuum*]	4E-58	423	2, 3
gb|AAU11490.1	monodehydroascorbate reductase I [*Pisum sativum*]	1E-65	491	2
gb|AAU11490.1	monodehydroascorbate reductase I [*Pisum sativum*]	2E-05	127	2
emb|CAC24549.1	glutathione S-transferase [*Cichorium intybus x Cichorium endivia*]	8E-05	288	3
emb|CAB53458.1	MnSOD [*Hevea brasiliensis*]*	1E-11	321	1
emb|CAC33844.1	putative CuZn-superoxide dismutase [*Populus tremula* x *Populus tremuloides*]	3E-40	406	3
gb|AAM97541.1	catalase 2 [*Capsicum annuum*]*	4E-11	397	2
emb|CAB56850.1	catalase [*Prunus persica*]*	2E-64	410	1
emb|CAC34417.1	Germin-like protein [*Pisum sativum*]	5E-30	380	2, 3
emb|CAC34417.1	Germin-like protein [*Pisum sativum*]	4E-32	368	3
Programmed Cell Death Inhibitor				
gb|AAR28754.1	Bax inhibitor [*Lycopersicon esculentum*]*	6E-31	350	2
emb|CAF74918.1	defender against cell death 1-like molecule [*Suberites domuncula*]	1E-12	412	2
Phenolic metabolism				
gb|AAP03017.1	4-coumarate-CoA ligase-like protein [*Arabidopsis thaliana*]	6E-10	501	1
ref|NP_564115.1	4-coumarate CoA ligase [*Arabidopsis thaliana*]	4E-12	114	3
gb|AAK28509.1	cinnamyl alcohol dehydrogenase [*Fragaria* x *ananassa*]	4E-07	401	1
gb|AAT39306.1	putative cinnamoyl-CoA reductase [*Solanum demissum*]	2E-57	510	2
gb|AAS48416.1	cinammate 4-hydroxylase [*Allium cepa*]	7E-51	362	3
emb|CAB71128.2	cationic peroxidase [*Cicer arietinum*]	6E-31	486	1, 3
emb|CAH69536.1	putative peroxidase [*Zinnia elegans*]	6E-10	117	2
emb|CAA71490.1	peroxidase [*Spinacia oleracea*]	E-100	947	2, 3
emb|CAA71492.1	peroxidase [*Spinacia oleracea*]	7E-35	376	3
emb|CAA71488.1	peroxidase [*Spinacia oleracea*]	6E-26	393	3
emb|CAB71128.2	cationic peroxidase [*Cicer arietinum*]	5E-77	471	3
Heat shock protein				
gb|AAQ24862.1	heat shock protein 90 [*Euglena gracilis*]*	2E-20	397	1, 3
gb|AAR18070.1	heat shock factor binding protein 2 [*Zea mays*]	4E-21	483	2
gb|AAR25848.1	17.5 kDa class I heat shock protein [*Carica papaya*]*	1E-15	409	2
emb|CAA44820.1	heat shock protein 70 [Nicotiana tabacum]*	1E-09	97	2
gb|AAQ08597.1	heat shock protein [*Hevea brasiliensis*]*	2E-30	407	3
Other stress response protein				
gb|AAP80290.1	resistance protein Tsu [*Arabidopsis thaliana*]	6E-12	325	2, 3
gb|AAP92753.1	stress-responsive protein [*Oryza sativa* (japonica cultivar-group)]	2E-07	467	2, 3
gb|AAP44394.1	nematode resistance-like protein [*Solanum tuberosum*]	3E-22	538	1
emb|CAB64227.1	disease resistance-like protein [*Arabidopsis thaliana*]	5E-16	479	2
gb|AAN17463.1	hypersensitive-induced reaction protein 2 [*Hordeum vulgare subsp. vulgare*]	3E-18	427	2
gb|AAD25626.1	Very similar to disease resistance proteins [*Arabidopsis thaliana*]	2E-14	383	2
gb|AAO12871.1	submergence induced protein 2-like [*Vitis vinifera*]	1E-19	182	2
emb|CAB79153.1	symbiosis-related like protein [*Arabidopsis thaliana*]*	3E-48	432	3
ref|XP_589501.1	Similar to ischemia/reperfusion inducible protein [*Bos taurus*]	8E-45	1118	1, 3
gb|AAO43165.1	Dehydration responsive element binding protein [*Gossypium hirsutum*]	4E-11	641	3
gb|AAR23528.1	metal tolerance protein 1; MTP1 [*Populus balsamifera*]	2E-28	444	4
gb|AAV71142.1	salt tolerance protein [*Sesuvium portulacastrum*]*	1E-67	429	3

gb|AAQ22345.1	BURP domain-containing protein [*Gossypium hirsutum*]	1E-24	235	2
emb|CAH59196.1	BURP-domain containing protein [*Plantago major*]	5E-32	313	2, 3
emb|CAA11428.1	alpha-hydroxynitrile lyase [*Manihot esculenta*]*	3E-10	354	2, 3
emb|CAA11428.1	alpha-hydroxynitrile lyase [*Manihot esculenta*]*	2E-25	358	2
emb|CAB79534.1	putative APG protein [*Arabidopsis thaliana*]	4E-39	416	3
emb|CAB79534.1	putative APG protein [*Arabidopsis thaliana*]	1E-21	217	3
emb|CAB79534.1	putative APG protein [*Arabidopsis thaliana*]	4E-20	357	3
emb|CAB79534.1	putative APG protein [*Arabidopsis thaliana*]	1E-38	589	3, 4
gb|AAT01926.1	putative coat protein [Pear black necrotic leaf spot virus]	7E-06	211	3
Allergen				
emb|CAB96876.2	pru p 1 [*Prunus persica*]	9E-06	380	1, 2
gb|AAV28626.1	Bet v I allergen [*Zea mays*]	7E-34	707	2, 3

* Homologous to Genbank sequences from *Hevea* latex.

† Number 1,2,3,4 and 5 represent respectively MDF180 6-72 hpi, MDF180 4-28 dpi, PB314 6-72 hpi, PB314 4-28 dpi and PB314 34-58 dpi libraries.

Figure 5 : Cell reactions of infected *M. ulei* leaflets of the partial resistant MDF180 cultivar and susceptible PB314 cultivars 10dpi. (A) MDF180 lesion with collapsed spongy parenchyma and upper dermal cells. In the palisade parenchyma, we note a granulated appearance of the cytoplasm (ve). (B) Starch grains (st) in MDF180 infected leaf. (C) PB314 infected leaf tissue presenting several apoptotic nucleus, some cell disruption and a clear cytoplasm. (D) Active nucleus in MDF180 infected leaf. hy : hyphae, con: conidiophore, sp: spore, ve : vesicule, nu: nucleus; st: starch grain; ap : apoptotic nucleus; ced : cell disruption.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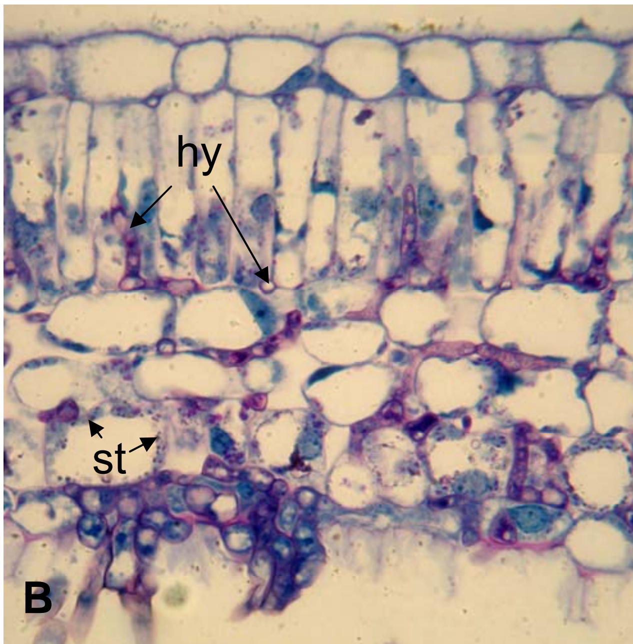
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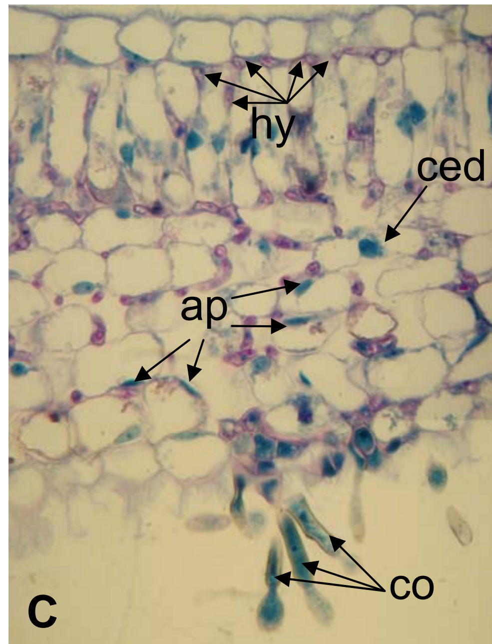
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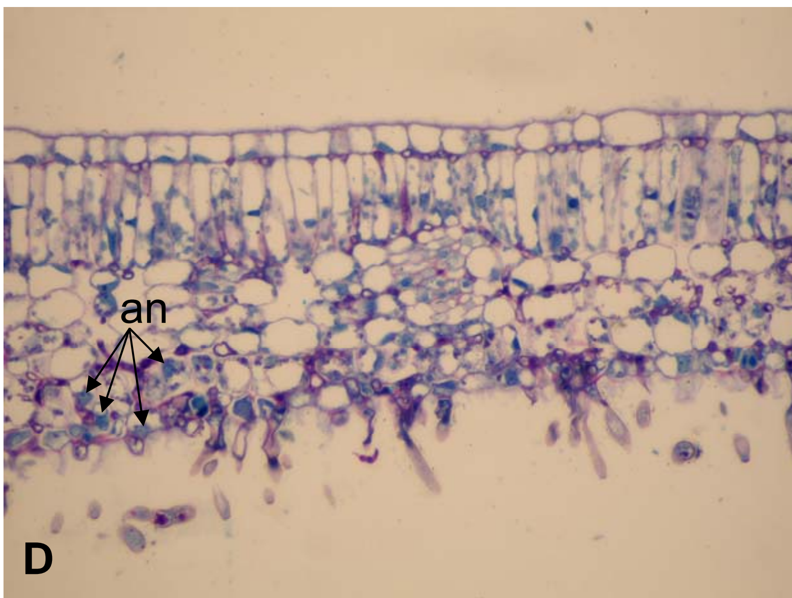
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