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454-pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* reveals *in planta*-expressed pathogen-secreted proteins and plant functions in a late compatible plant–rust interaction

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SUMMARY

Coffee (*Coffea arabica* L.), one of the key export and cash crops in tropical and subtropical countries, suffers severe losses from the rust fungus *Hemileia vastatrix*. The transcriptome of *H. vastatrix* was analysed during a compatible interaction with coffee to obtain an exhaustive repertoire of the genes expressed during infection and to identify potential effector genes. Large-scale sequencing (454-GS-FLEX Titanium) of mixed coffee and rust cDNAs obtained from 21-day rust-infected leaves generated 352 146 sequences which assembled into 22 774 contigs. In the absence of any reference genomic sequences for *Coffea* or *Hemileia*, specific trinucleotide frequencies within expressed sequence tags (ESTs) and BLAST homology against a set of dicots and basidiomycete genomes were used to distinguish pathogen from plant sequences. About 30% (6763) of the contigs were assigned to *H. vastatrix* and 61% (13 951) to *C. arabica*. The majority (60%) of the rust sequences did not show homology to any genomic database, indicating that they were potential novel fungal genes. *In silico* analyses of the 6763 *H. vastatrix* contigs predicted 382 secreted proteins and identified homologues of the flax rust haustorially expressed secreted proteins (HESPs) and bean rust transferred protein 1 (RTP1). These rust candidate effectors showed conserved amino-acid domains and conserved patterns of cysteine positions suggestive of conserved functions during infection of host plants. Quantitative reverse transcription-polymerase chain reaction profiling of selected rust genes revealed dynamic expression patterns during the time course of infection of coffee leaves. This study provides the first valuable genomic resource for the agriculturally important plant pathogen *H. vastatrix* and the first comprehensive *C. arabica* EST dataset.

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INTRODUCTION

The basidiomycete *Hemileia vastatrix* Berk. & Broome 1869 (Berkeley, 1869) (order Pucciniales) is an obligatory parasite specific to coffee plants (*Coffea* spp.) (Bettencourt and Rodrigues, 1988) and causes coffee leaf rust, one of the most destructive diseases of the cash crop *Coffea arabica* L. The fungus is widely distributed in all regions of the world in which coffee is grown and can represent a serious limitation to economically sustainable coffee production (Bettencourt and Rodrigues, 1988). The natural resistance of coffee plants to leaf rust is conditioned by gene-for-gene interactions, with at least nine plant resistance factors that are implicated in the recognition of the corresponding virulence genes in *H. vastatrix* isolates (Bettencourt and Rodrigues, 1988; Rodrigues *et al*., 1975). Little information is available on the fungal genetic factors that condition coffee–rust interactions. To date, 45 *H. vastatrix* races have been characterized according to their virulence spectrum on a set of 18 coffee differentials, with the number and pattern of occurrence varying by region (Várzea and Marques, 2005). In the last few years, new rust races have overcome the resistance of some improved cultivars grown in several countries (Silva *et al*., 2006; Várzea and Marques, 2005). Because the life cycle of *H. vastatrix* is essentially asexual, via cyclic uredospore production on host leaves (Rodrigues *et al*.,

1975), no genetic assumption could be derived for the identification of loci carrying rust avirulence genes.

The initiation of the dikaryotic phase of *H. vastatrix* on coffee leaves involves specific events, including appressorium formation over stomata, prior to progression through a defined set of developmental stages required to enter stomatal openings and to form the first haustoria within stomatal subsidiary cells. Further on, the infection process involves the colonization of mesophyll cells by intercellular hyphal growth and intense haustoria formation, and sporulation can start as early as 20 days after infection (Coutinho *et al*., 1993; Martins and Moraes, 1996; Ramiro *et al*., 2009; Rodrigues *et al*., 1975; Silva *et al*., 1999, 2002). Histological observations have shown that *C. arabica* resistance is expressed by a hypersensitive response (HR) with cell death of stomatal and mesophyll cells (Martins and Moraes, 1996; Silva *et al*., 2002, 2008). Molecular analyses have indicated that perception of the fungus, whether virulent or avirulent, occurs when the pathogen enters the stomata (Ganesh *et al*., 2006; Ramiro *et al*., 2009). However, specific host resistance responses, including hypersensitive cell death, H_2O_2 production and defence-related gene expression patterns, are associated with the production of haustoria in mesophyll cells, and not with the production of haustoria in stomatal cells, suggesting that specific recognition of *H. vastatrix* occurs at a later stage (Ramiro *et al*., 2009).

Recently, major insights have emerged from studies on biotrophic fungi, indicating that they secrete effector proteins, including virulence and avirulence proteins, that alter host physiology and defence responses (for review, see Stergiopoulos and de Wit, 2009). Effector proteins may be subdivided into two broad categories depending on whether they are secreted in the apoplasm or delivered into the cytoplasm of the host cell. In the flax rust fungus *Melampsora lini*, the avirulent genes identified so far encode small secreted proteins, are highly expressed in the haustoria and their products are recognized by flax (*Linus usitatissimum*) resistance proteins inside plant cells (Catanzariti *et al*., 2006; Dodds *et al*., 2004; Rafiqi *et al*., 2010). Flax rust avirulence proteins have no homology to any known protein (Catanzariti *et al*., 2006), but homologues have been identified recently in the related species *Melampsora larici-populina* (Hacquard *et al*., 2010). In the same way, a family of rust transferred proteins (RTPs) has been identified that is conserved in rust fungi (Joly *et al*., 2010; Puthoff *et al*., 2008). The RTP1 protein was primarily identified in *Uromyces fabae*, where it translocates from the haustoria into the host and accumulates in the nucleus (Kemen *et al*., 2005). Searches in public protein databases for other rust secreted proteins identified the *Cronartium ribicola* secreted protein CrorI (Ekramoddoullah *et al*., 1999) and several *Melampsora* spp. proteins (Feau *et al*., 2007; Hacquard *et al*., 2010; Joly *et al*., 2010).

Until recently, the identification of *in planta*-expressed transcripts was a major limitation to studies of biotrophic pathogens. Sequencing through the Sanger approach has led to the identification of several hundreds of genes and a few secreted proteins (Catanzariti *et al*., 2006; Hahn and Mendgen, 1997; Hu *et al*., 2007; Joly *et al*., 2010; Puthoff *et al*., 2008; Yin *et al*., 2009). The fundamental knowledge of the functional genome is now being enhanced by the ability to deeply probe an organism's transcriptome using high-throughput sequencing data production. In this study, we used 454 massively parallel sequencing to gain new insights into the *in planta* transcriptome of the coffee rust parasite. These data reveal the first features of the *H. vastatrix* transcriptional landscape to establish a catalogue of putative fungal effector genes and to significantly advance our understanding of the coffee–rust fungus interaction.

RESULTS

454-pyrosequencing of transcripts from *Coffea* leaves infected by *H. vastatrix*

A total of 352 146 nucleotide reads (118 113 878 nucleotides) was generated by single read 454-pyrosequencing and assembled into 22 774 contigs (Table 1; Data S1, see Supporting Information) by Newbler (v2.0.00.22). Remaining unassembled reads (65 428) correspond to low-quality sequences rejected during the assembly process and putative singleton sequences. These singletons were not considered further in this study. Assembled contigs ranged between 85 and 4589 base pairs (bp) and consisted of 2–421 assembled reads. Contigs were commonly smaller than 500 bp (64%), but 11% were larger than 1 kb, indicating that the 454-approach was useful for the generation of large transcript sequences. In addition, 30% of the contigs (6804) contained 10 reads or more. An estimation of the relative abundance of contigs (number of reads relative to the contig length) showed that 10% (2365 contigs) represented

Table 1 Number of 454-reads and contigs generated by 454-GS-FLEX Titanium pyrosequencing from rust-infected *Coffea arabica* leaves.

	Total	Fungal	Plant
Contig no.	22 774	6 763	13 951
Read no.	286 718	57 332	205 089
Average size (bp)	542	426	631
Contig no. with:			
Size ≥ 1 kb	2 593	373	2 198
Content ≥ 10 reads	6 804	1 168	5 120
Content ≥ 50 reads	1 038	163	790
Content ≥ 200 reads	53	8	38
Ra ≥ 1	73	9	49
Ra ≥ 0.1	1 134	259	643
Ra ≥ 0.05	2 381	533	1 418

Ra, relative abundance (Ra = number of reads/contig length).

transcripts with a medium to high rate of expression (Ra > 0.05) from rust-infected *Coffea* leaf tissues (Table 1).

Origin of expressed sequence tags (ESTs) from infected leaf tissues

A strategy based on parallel prediction approaches (Fig. 1) was set to determine the origin of plant and fungal transcripts from rust-infected *C. arabica* leaf tissues. In total, six different prediction methods were used to determine the plant or fungal origin of the *Coffea*/*Hemileia* contig sequences. In a first step, specific trinucleotide frequencies were estimated for *Coffea* and *Hemileia* transcribed sequences with the MIPS EST3 program (Emmersen *et al*., 2007). The EST3 program was specifically trained with an intrinsic training set of *Coffea* and Pucciniales sequences from public databases [see Experimental procedures and Fig. S1 (Supporting Information)]; however, the program indicated that the dinucleotide bias between *Coffea* and Pucciniales was not sufficiently high to provide the most accurate prediction. Such a low bias between plant and fungal sequences has been reported previously between the model species *Arabidopsis thaliana* and *Saccharomyces cerevisiae* (Gentles and Karlin, 2001). A second step involved homology searches against public databases or selected sequence databases generated for this study [see Experimental procedures and Fig. S2 (Supporting Information)]. BLAST homology searches always predicted a larger number of plant contigs, whereas trinucleotide frequency-based predictions always identified a larger number of fungal sequences (Figs S1 and S2), which could reflect the smaller number of genetic resources for rusts relative to plants in databases. The final set of tentative *C. arabica* and *H. vastatrix* contigs was determined on the basis of the number of plant or fungus predictions (see Experimental procedures). At the end, 13 951 contigs were attributed to the plant host *C. arabica*, and 6763 contigs were attributed to the rust fungus *H. vastatrix* (Tables 1 and S1, see Supporting Information). An additional set of 2060 contigs (9% of the total number of contigs) could not be attributed to *C. arabica* or *H. vastatrix* because they either showed a similar number of plant and fungus predictions (1358 contigs not resolved) or none of the prediction methods was able to determine their origin (702 contigs not determined).

Cellular category distribution of plant and fungal contigs

Contigs attributed to *C. arabica* and *H. vastatrix* were compared with the euKaryotic Orthologous Group (KOG) database in order to classify sequences into functional cellular categories (Fig. 2). Almost half of the sequences (43% and 46% of plant and fungus, respectively) had no significant matches at the cut-off *E*-value of $<10^{-5}$. In the plant contigs, the most important cellular categories corresponded to the following: signal transduction mechanisms (7.2%); post-translational modification, protein turnover and chaperones (7.1%); carbohydrate transport and metabolism (3.8%); and intracellular trafficking, secretion and vesicular transport (3.7%). The proportion of plant transcripts falling into these categories probably reflects the strong impact of rust growth in plant living tissues, including remobilization of plant photoassimilates and manipulation of host defence. In the fungal contigs, the most important cellular categories corresponded to the following: post-translational modification, protein turnover and chaperones (6.6%); translation, ribosomal structure and biogenesis (5.8%); energy production and conversion (4.3%); intracellular trafficking, secretion and vesicular transport (3.9%); and signal transduction mechanisms (3.7%). The proportions of fungal sequences falling into amino acid transport and metabolism, lipid transport and metabolism, and carbohydrate transport and metabolism categories were also notable (Fig. 2). The high representation of transcripts falling into translation and post-translation categories highlights the fact that fungal cells are highly active at this stage of infection, which probably reflects the switch from an exclusively biotrophic-oriented phase to a sporulation phase devoted to both the functioning of the haustorial biotrophic structures and the production of uredospores in uredinia, as observed in other rust fungi (Hacquard *et al*., 2010).

Coffea arabica transcripts expressed during the late compatible interaction with the coffee rust fungus

The 13 951 *C. arabica* contigs represented 71% of the reads, with an average length of 631 bp (Table 1). Indeed, most of the larger contigs (85%) identified in the assembly were attributed to the plant host. Thirty eight contigs contained more than 200 reads, representing highly expressed plant transcripts (Table 2). Interestingly, 95% of the plant contigs showed homology in the GenBank nonredundant nucleotide (nr) database, but only 55% had a significant BLAST hit against *Coffea* ESTs deposited in dbEST. As the contigs were generally shorter than the complete coding sequence, distinct contigs matching the same gene were commonly found. In total, 6202 plant contigs may correspond to novel coffee transcripts not yet described. For instance, 13 new *WRKY* transcription factor genes were found (data not shown) that have not been reported previously in coffee (Ramiro *et al*., 2010). To further investigate the extent of transcriptomic coverage in our experiment, we investigated the presence of known genes in various metabolic pathways, such as the caffeine or chlorogenic acid biosynthesis pathways, which are known to be specific for coffee and a few plant species (Ashihara *et al*., 2008; Mahesh *et al*., 2007),

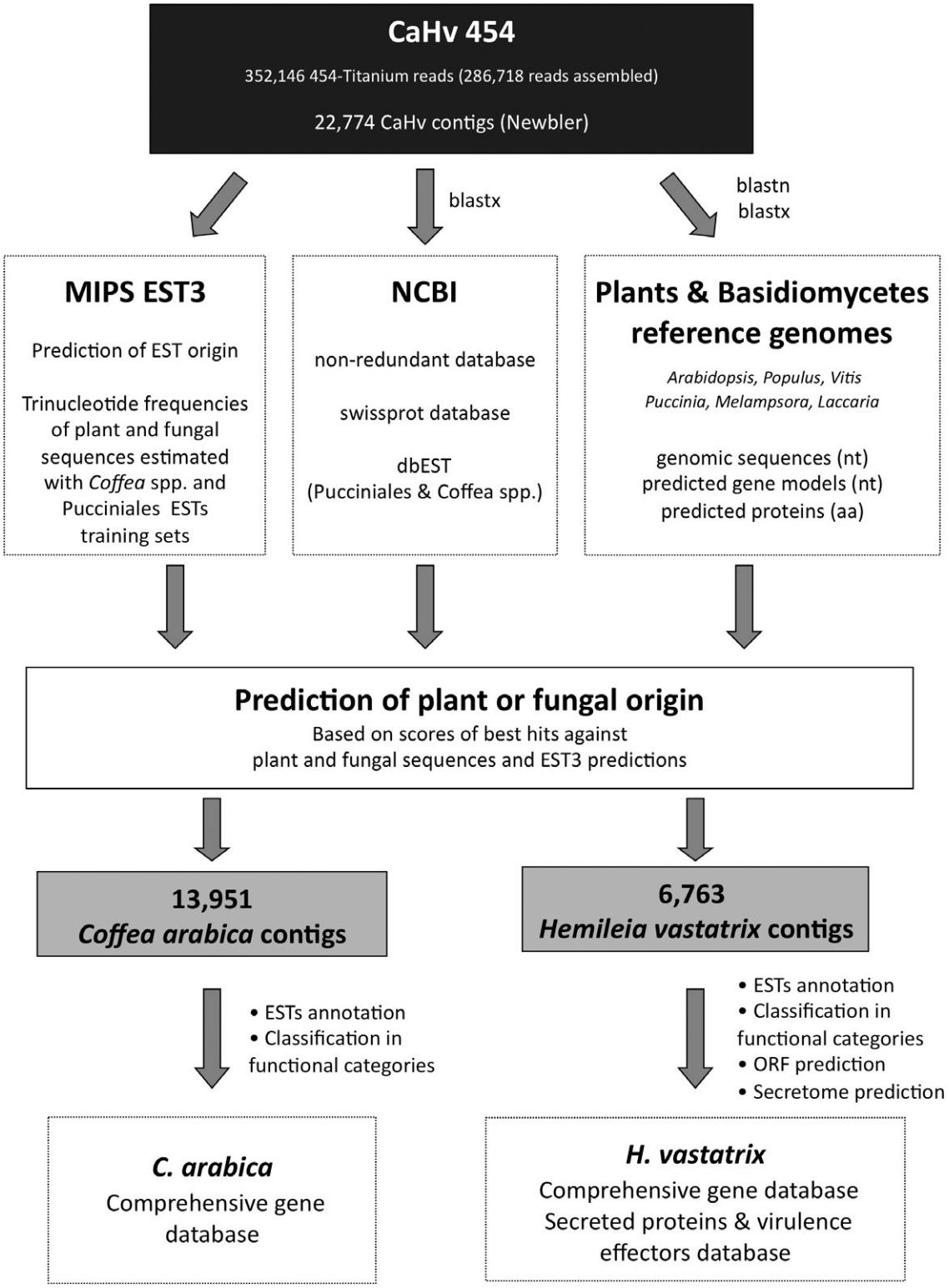


Fig. 1 Bioinformatic procedure used to determine the origin of sequences produced by the 454-GS-FLEX Titanium pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix*.

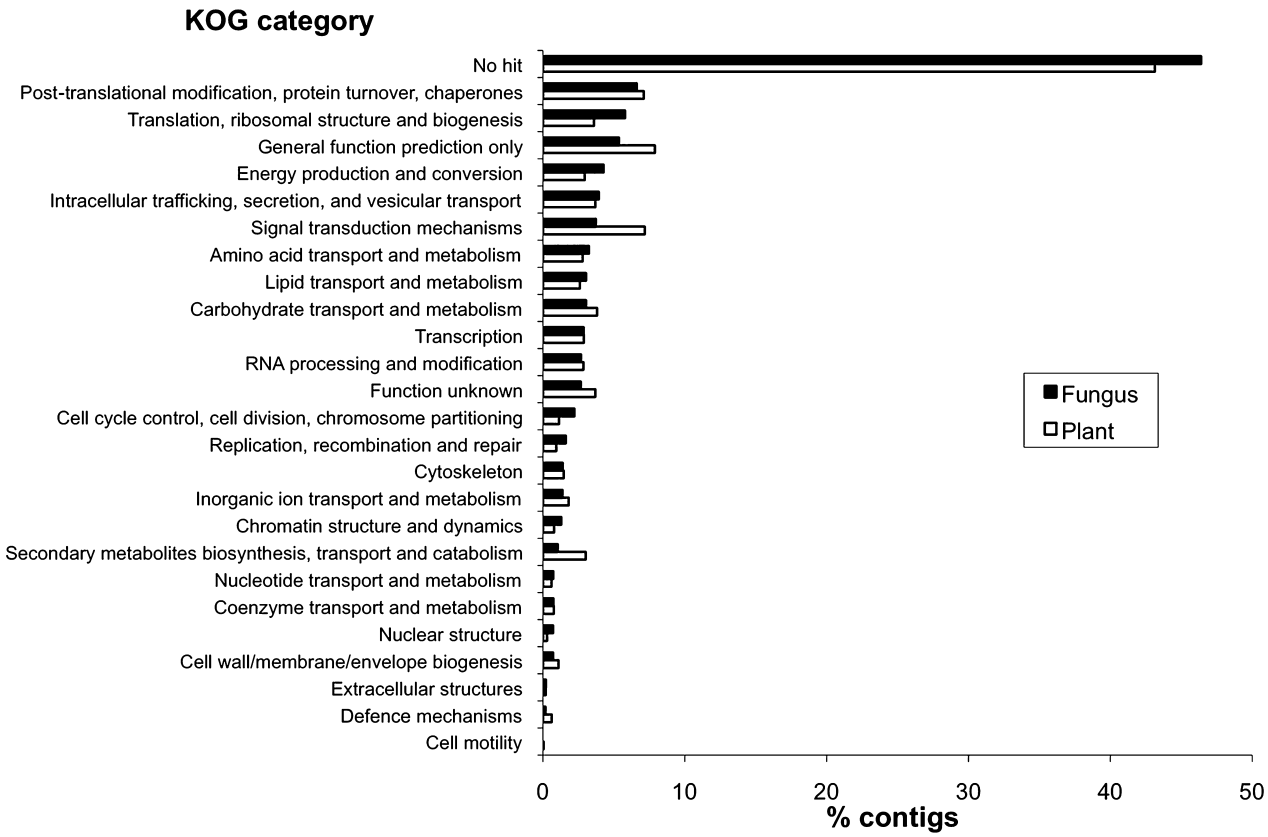


Fig. 2 Assignment of fungal and plant sequences to cellular categories based on their KOG (euKaryotic Orthologous Group) classification.

and the photosynthesis and respiratory pathways, known to be altered in plant–rust interactions (Major *et al*., 2010). All the genes involved in the metabolic pathways were retrieved in the *C. arabica* contigs, whereas only 70% of the caffeine or chlorogenic acid biosynthesis pathways were found. Genes involved in glycolysis and the tricarboxylic acid (TCA) cycle were represented by several abundant contigs, with up to 250 reads (Ra = 0.75) for contigs encoding the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Tables 2 and S1), indicating that glycolysis is probably activated in rust-infected leaves. A total of 532 contigs fell into the carbohydrate transport and metabolism category and 390 contigs fell into the amino acid transport and metabolism category, which highlights that plant metabolism is highly active at this stage of rust infection. In addition, contigs encoding functions related to the plant defence response were among the most abundant contigs (Tables 2 and S1), such as the pathogenesis-related (PR) proteins (1,3-β-glucanases, PR1b, PR-5 of the thaumatin-like protein family and chitinases) known to accumulate at a late stage in compatible plant–rust interactions (Duplessis *et al*., 2009; Fernandez *et al*., 2004; Ramiro *et al*., 2009).

Hemileia vastatrix transcripts expressed during late infection of *C. arabica* leaf tissues

The 6763 *H. vastatrix* contigs showed an average length of 426 bp, but only a few contigs (5.5%) were larger than 1 kb, indicating that fungal contigs were enriched in smaller sequences relative to the total set of contigs (Table 1). About 17% of the contigs were composed of a minimum of 10 reads, indicating that the infected *C. arabica* leaf tissues contained a number of rust transcripts that were substantially expressed (Table 2). A small number of *H. vastatrix* contigs had hits in the nr or Swiss-Prot databases (24%), but 33% showed homology to gene models predicted in *M. larici-populina* or *Puccinia graminis* f.sp. *tritici*, the two rust genomes sequenced so far. Only 24% of the *H. vastatrix* contigs presented a hit against *Laccaria bicolor*, the basidiomycete with the largest number of genes predicted so far (Martin *et al*., 2008). Among the contigs that showed homology with a rust gene, but not with *L. bicolor* genes, 521 showed no homology against any other database, probably representing Pucciniales lineage-specific genes not shared across the Basidiomycota phyllum. Interestingly, 26 contigs did not show homology with predicted rust gene models, but presented a significant

Table 2 Most abundant 454-contigs of *Coffea arabica* and *Hemileia vastatrix* expressed in rust-infected *Coffea arabica* leaves at 21 days post-inoculation.

Contig ID	Length*	Reads*	Best BLAST hit (species)†	Accession no.	*E*-value	Best RPSBLAST hit (KOG)‡	KOG category‡
Fungal contigs							
Contig00342	124	407	No hit			No hit	No hit
Contig19064	150	298	No hit			No hit	No hit
Contig00300	1769	247	Translation elongation factor EF-1α (*Candida albicans*)	XP_711899.1	0.0	Translation elongation factor EF-1α/Tu	Translation, ribosomal structure and biogenesis
Contig18071	513	245	No hit			No hit	No hit
Contig19429	256	231	No hit			RhoA GTPase effector DIA/diaphanous	Signal transduction mechanisms, cytoskeleton
Contig20014	256	212	No hit			No hit	No hit
Contig04616	1604	202	Aryl-alcohol dehydrogenase (*Aspergillus terreus*)	XP_001217691.1	E-108	Voltage-gated shaker-like K+ channel, subunit β/KCNAB	Energy production and conversion
Contig19637	1576	201	Hypothetical protein AAur_0776 (*Arthrobacter aurescens*)	YP_946576.1	4.00E-26	No hit	No hit
Contig00263	339	197	No hit			No hit	No hit
Contig19425	352	197	No hit			No hit	No hit
Contig00144	479	189	No hit			No hit	No hit
Contig00025	278	186	No hit			No hit	No hit
Contig17859	114	182	No hit			No hit	No hit
Contig00063	263	182	No hit			No hit	No hit
Contig20059	1031	179	No hit			No hit	No hit
Contig19461	422	178	No hit			No hit	No hit
Contig20013	611	178	No hit			No hit	No hit
Contig00486	574	174	No hit			No hit	No hit
Contig21472	918	172	Unknown protein (*Populus trichocarpa* × *Populus deltoides*)	ABK96247.1	1.00E-36	No hit	No hit
Contig16131	398	171	No hit			RNA polymerase II, large subunit	Transcription
Contig18436	117	169	No hit			No hit	No hit
Contig17838	366	166	No hit			No hit	No hit
Contig00104	264	164	No hit			No hit	No hit
Contig18196	1345	164	Thiamine biosynthesis protein NMT1, plant-induced rust protein 1 (*Uromyces fabae*)	O00057	E-162	No hit	No hit
Contig00082	126	159	No hit			No hit	No hit
Plant contigs							
Contig21649	2095	421	Os03g0708600 (*Oryza sativa*)			Nuclear pore complex, Nup98 component	Nuclear structure, intracellular trafficking, secretion and vesicular transport
Contig00026	123	393	β-1,3-Glucanase, basic (*Coffea arabica* × *Coffea canephora*)	AAQ90286.1	2.00E-16	No hit	No hit
Contig21461	440	392	Metallothionein-like protein MT-1 (*Mimulus guttatus*)	P20238	2.00E-18	No hit	No hit
Contig21497	1471	349	Os03g0708600 (*Oryza sativa*)	CAO23566.1	0.0	No hit	No hit
Contig17133	389	310	Pathogenesis-related thaumatin-like protein (*Coffea arabica*)	ABW76502.1	2.00E-75	No hit	No hit
Contig19448	100	299	Thaumatin-like protein (*Sambucus nigra*)	AAK59275.1	3.00E-07	No hit	No hit
Contig18008	157	290	Asparagine synthetase (*Helianthus annuus*)	AAF02775.1	1.00E-12	Asparagine synthase (glutamine hydrolysing)	Amino acid transport and metabolism
Contig18133	802	285	Os03g0708600 (*Oryza sativa*)	Q42679|	E-112	*S*-Adenosylmethionine decarboxylase	Signal transduction mechanisms
Contig19562	632	280	Polyubiquitin [*Pinus sylvestris*]	CAA66667.1	E-107	Ubiquitin/40S ribosomal protein S27a fusion	Translation, ribosomal structure and biogenesis

Table 2 *Continued*.

Contig ID	Length*	Reads*	Best BLAST hit (species)†	Accession no.	*E*-value	Best RPSBLAST hit (KOG)‡	KOG category‡
Contig18010	115	276	Unknown [*Populus trichocarpa*]	ABK95431.1	4.00E-14	Asparagine synthase (glutamine hydrolysing)	Amino acid transport and metabolism
Contig17878	1169	276	Photosystem II CP47 chlorophyll apoprotein (*Dioscorea elephantipes*)	YP_001294378.1	7.00E-43	No hit	No hit
Contig01991	479	269	Os03g0708600 (*Oryza sativa*)	CAO62471.1	8.00E-28	No hit	No hit
Contig18005	135	268	Asparagine synthetase (*Elaeis guineensis*)	ACF06573.1	1.00E-05	No hit	No hit
Contig17908	130	266	B-Xylosidase (*Camellia sinensis*)	ACD93208.1	2.00E-13	No hit	No hit
Contig00069	107	260	Glucan endo-1,3-β-D-glucosidase (*Solanum lycopersicum*)	CAA52871.1	1.00E-10	No hit	No hit
Contig19395	344	256	Hypothetical protein OsI_015840 (*Oryza sativa*)	EAY94607.1	7.00E-52	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Carbohydrate transport and metabolism
Contig17879	361	256	Photosystem II CP47 chlorophyll apoprotein (*Coffea arabica*)	YP_817508.1	8.00E-66	No hit	No hit
Contig18062	575	252	Os03g0708600 (*Oryza sativa*)	BAD91081.1	4.00E-61	β-Galactosidase	Carbohydrate transport and metabolism
Contig21900	1869	250	Hypothetical protein (*Vitis vinifera*)	CAN81585.1	0.0	3-oxoacyl CoA thiolase	Lipid transport and metabolism
Contig18097	103	248	Unknown (*Populus trichocarpa*)	ABK94055.1	5.00E-08	No hit	No hit
Contig20631	728	244	Os03g0708600 (*Oryza sativa*)	CAO43774.1	E-112	Asparaginyl peptidases	Post-translational modification, protein turnover, chaperones
Contig21491	218	237	Unknown (*Populus trichocarpa*)	ABK95810.1	4.00E-24	No hit	No hit
Contig21248	747	237	Os03g0708600 (*Oryza sativa*)	CAO39242.1	2.00E-88	Asparagine synthase (glutamine hydrolysing)	Amino acid transport and metabolism
Contig17869	274	235	Unnamed protein product (*Vitis vinifera*)	CAO45976.1	1.00E-17	Iron/ascorbate family oxidoreductases	Secondary metabolite biosynthesis, transport and catabolism
Contig00669	140	234	Unknown protein (*Arabidopsis thaliana*)	NP_194974.1	6.00E-05	No hit	No hit

*Length of contigs in nucleotides and number of reads in the assembled contigs.

†BLASTX searches were carried out against the National Center for Biotechnology Information (NCBI) nonredundant nucleotide (nr) database.

‡RPSBLAST searches were carried out against the euKaryotic Orthologous Genes (KOG) database.

match with *M. larici-populina* and/or *P. graminis* f.sp. *tritici* genome sequences. These *H. vastatrix* contigs probably correspond to mis-predicted rust genes in the corresponding genomes, and may be helpful in annotating new genes in the reference rust genomes. Finally, among the 6763 *H. vastatrix* contigs, 60% showed no homology to the databases used in this study and may correspond to genes unique to coffee rust. In contrast, several homologues of previously described rust transcripts associated with the haustorial infection structure were found among the most abundant *H. vastatrix* transcripts (Table 2), such as the NMT1 protein, a NADP-dependent mannitol dehydrogenase, the thiamine synthesis-related enzyme THI4 and a plasma membrane (H+)-ATPase described in *U. fabae* (Hahn and Mendgen, 1997). Other homologues of *in planta* bean rust transcripts previously reported to be expressed in haustoria were detected among *H. vastatrix* transcripts of lower abundance, notably several amino acid transporters and the hexose transporter HXT1 (Voegele *et al*., 2001). Several secreted proteins of low molecular weight previously described in rust fungi were also among the most abundant *H. vastatrix* transcripts (see below). Signalling genes were also present among abundant contigs, encoding 14-3-3 proteins, calcium and calmodulin-related protein kinases or G-proteins. All these results tend to indicate that *H. vastatrix* is transcriptionally very active at this stage of coffee leaf infection, including both biotrophic structures (i.e. haustoria) and the onset of sporulation.

A total of 22 contigs predicted to be of *H. vastatrix* origin, including 12 genes with known homology [heterotrimeric G-protein α subunit (Gp-α); mitogen-activated protein kinase (MAPK); chitin deacetylases (CD1 and CD2); amino acid

transporters (AAT1_2 and AAT3); aspartate amino-transferase (Asp_AT); invertase (Inv); mannitol dehydrogenase (MAD); translation elongation factor (TEF); β-tubulin (β-Tub); cytochrome B (CytB)], three Pucciniales-conserved genes (RTP; haustorially expressed secreted proteins HESP-379 and HESP-C49) and seven new rust genes with no known homology in international databases, was selected in order to test the accuracy of the bioinformatic selection and confirm the fungal origin of the contigs. Polymerase chain reaction (PCR) analyses conducted on *C. arabica* and *H. vastatrix* DNA confirmed the fungal origin of the contigs (Fig. S3, see Supporting Information).

Prediction of secreted proteins encoded by *H. vastatrix* transcripts

Using stringent criteria, bioinformatic analyses predicted a total of 382 rust contigs encoding putative secreted proteins in the *H. vastatrix* sequence dataset. In detail, 142 showed homology against basidiomycete predicted gene models or nr and Swiss-Prot databases, 86 against Pucciniales ESTs and 154 had no homology in selected databases. Among the 382 *H. vastatrix* contigs encoding secreted proteins, 50 contained 30 or more reads supportive of a high expression in infected leaf tissues (Tables 3 and S2, see Supporting Information). Among them, a homologue of the previously described *U. fabae* secreted protein RTP1 (Kemen *et al*., 2005) was found, and some genetic determinants often related to the virulence of fungal pathogens, such as cyclophilin or different types of carbohydrate active enzyme (chitinases, endoglucanases) (Table 3). It is tempting to speculate that specific coffee rust effectors reside in the set of predicted secreted proteins that showed no homology with any other rusts or fungi. Among the 382 predicted protein sequences, 20 encoded small proteins of less than 200 amino acids highly enriched in cysteine (Cys) residues (between 5% and 10% of all amino acids) (Table S2).

Identification of conserved rust candidate effectors

Hemileia vastatrix transcripts encoding secreted proteins were compared across the order Pucciniales (rust ESTs and genome sequences) and against the Plant–Host Interaction database (PHI-base) to determine conserved virulence-related genes in plant pathogens and candidate effectors among rust fungi (Table S3, see Supporting Information). The absence of homology in a given EST set may be related to a lack of expression in a particular physiological state and not to the absence of the corresponding gene in the rust species. Interestingly, several *in planta*-expressed secreted proteins reported in *U. fabae* and *M. lini* were identified in several rust species, including *H. vastatrix* and/or *M. larici-populina* and *P. graminis* f.sp. *tritici* (Table S3). The sequences of the homologues of *U. fabae* RTP1 (Kemen *et al*., 2005) and *M. lini* HESP-C49, HESP-178 and HESP-379 (Catanzariti *et al*., 2006), representing haustorially expressed rust effectors found in several rust fungi, were selected and aligned. The alignment of RTP1 homologues (Fig. 3), including the RTP homologues described previously in *Uromyces* spp. and *P. graminis* (Puthoff *et al*., 2008), three *H. vastatrix* RTP homologues and 10 *Melampsora* spp. RTP homologues, highlighted species-related divergence with a rather conserved region of 130 amino acids at the C-terminus. In contrast, the N-terminal region of RTP-like proteins was relatively divergent between rust species (Fig. 3). HESP homologues identified in several rust species, including *H. vastatrix*, did not present strong similarity along the protein sequence; however, the Cys positions were highly conserved in homologues of *M. lini* HESP-C49 and HESP-178, with 11 and seven conserved Cys residues, respectively (Fig. 4). HESP-178 contains the 8-Cys CFEM (common in fungal and extracellular membrane) domain previously described in the G-protein-coupled receptor Pth11 and required for virulence in *Magnaporthe oryzae* (Kulkarni *et al*., 2003); however, the 8-Cys composing the CFEM domain was only found in *M. lini*, *M. larici-populina* and *H. vastatrix* homologues (Fig. 4). Rust homologues of HESP-379 (Fig. 4) showed a highly conserved region of 100 amino acids following the peptide signal and a less conserved C-terminal region enriched in serines that contains a predicted site for the glycosylphosphatidylinositol (GPI) anchor, which indicates that this protein probably remains attached to the exterior leaflet of the fungal plasma membrane (Pierleoni *et al*., 2008). Finally, comparison with the PHI-base identified nine contigs for which homologous genes in other plant or animal pathogenic fungi had a referenced phenotype related to virulence (Table S3). For instance, two contigs matched the *Botrytis cinerea bcpme1* gene encoding a pectin methylesterase gene, the disruption of which results in reduced virulence on apple fruits, grapevine and *A. thaliana* leaves (Valette-Collet *et al*., 2003). Another contig showed homology to the *Magnaporthe grisea GAS1* gene encoding a small protein expressed specifically during appressorium formation, with a role in rice leaf penetration (Xue *et al*., 2002).

Expression profiling of *H. vastatrix* genes during infection of coffee leaves

Taking advantage of the genomic resource generated for *H. vastatrix*, specific primers were designed from the 22 rust gene sequences selected previously (Fig. S3) to quantify their transcript accumulation by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) during the time course of infection of *C. arabica* leaves. Fungal gene expression profiles in coffee leaves were assessed at 18 and 48 h post-inoculation (hpi) and at 7 and 21 days post-inoculation (dpi). The later time point corresponds to the infection stage used for

Table 3 Fifty most abundant rust transcripts of *Hemileia vastatrix* encoding putative secreted proteins.

Contig ID	Length*	Reads*	Best BLAST hit (species)†	Accession no.	*E*-value	Best BLAST hit against Mlp SP (protein ID)‡	*E*-value
Contig20014	256	212	No hit			No hit	
Contig19637	1576	201	Hypothetical protein AAur_0776 (*Arthrobacter aurescens*)	YP_946576.1	4.00E-26	112808	8.00E-22
Contig00486	574	174	No hit			No hit	
Contig21472	918	172	Unknown (*Populus trichocarpa* × *Populus deltoides*)	ABK96247.1	1.00E-36	91075	1.00E-44
Contig00104	264	164	No hit			No hit	
Contig00329	1293	149	No hit			85997	1.00E-17
Contig22018	1021	148	Rust transferred protein (*Melampsora occidentalis*)	ABS86407.1	3.00E-13	No hit	
Contig00379	943	140	Galactan 1,3-β-galactosidase (*Phanerochaete chrysosporium*)	BAD98241.1	2.00E-66	62953	4.00E-25
Contig23035	551	123	Hypothetical protein (*Cryptococcus neoformans*)	XP_569628.1	2.00E-18	78206	4.00E-43
Contig00017	706	121	No hit			No hit	
Contig01344	1133	120	No hit			77195	2.00E-16
Contig19484	2442	115	AF353616_1 Cro r II (*Cronartium ribicola*)	AAK28629.1	0.0	43507	0.0
Contig20033	189	115	No hit			No hit	
Contig21820	1318	101	No hit			No hit	
Contig21571	850	100	No hit			No hit	
Contig17858	1053	97	No hit			No hit	
Contig20611	1593	94	Hypothetical protein UM02377.1 (*Ustilago maydis*)	XP_758524.1	9.00E-87	No hit	
Contig18013	1370	82	Phospholipase/lecithinase/haemolysin (*Burkholderia dolosa)*	YP_002099763.1	4.00E-14	68520	2.00E-39
Contig21062	105	78	No hit			No hit	
Contig22188	1339	75	No hit			No hit	
Contig03126	1280	70	Predicted protein (*Coprinopsis cinerea*)	XP_001830463.1	8.00E-37	No hit	
Contig22364	748	64	No hit			No hit	
Contig00659	1891	63	Hypothetical protein An14g01620 (*Aspergillus niger*)	XP_001400789.1	2.00E-69	95653	8.00E-43
Contig18607	1578	58	Endopeptidase (*Cryptococcus neoformans*)	XP_566887.1	E-137	42703	0.0
Contig04624	1283	56	No hit			104797	6.00E-09
Contig04096	803	56	No hit			104797	1.00E-17
Contig05042	1348	53	Hypothetical protein CIMG_08209 (*Coccidioides immitis*)	XP_001241046.1	4.00E-75	No hit	
Contig02757	1368	51	Predicted protein (*Laccaria bicolor*)	XP_001874996.1	2.00E-30	72374	7.00E-41
Contig18226	1260	50	Pectinesterase family protein (*Neosartorya fischeri*)	XP_001262221.1	3.00E-22	75334	2.00E-18
Contig00575	739	49	No hit			No hit	
Contig03936	1515	48	Hypothetical protein (*Melampsora medusae* f.sp. *deltoidis*)	ABS86355.1	5.00E-29	71512	3.00E-35
Contig03932	2212	48	Predicted protein (*Laccaria bicolor*)	XP_001878325.1	4.00E-07	70885	7.00E-54
Contig02027	2008	46	Hypothetical protein CC1G_00344 (*Coprinopsis cinerea*)	XP_001837208.1	E-127	116816	E-146
Contig06274	1187	45	Unknown (*Populus trichocarpa* × *Populus deltoides*)	ABK96247.1	2.00E-31	86274	3.00E-35
Contig04146	665	45	No hit			No hit	
Contig20800	1878	44	Hypothetical protein UM04400.1 (*Ustilago maydis*)	XP_760547.1	E-108	44205	E-163
Contig18836	1255	42	Endoglucanase (*Phanerochaete chrysosporium*)	AAU12275.2	1.00E-49	47207	6.00E-71
Contig00581	1427	42	Hypothetical protein PTRG_11055 (*Pyrenophora tritici-repentis*)	XP_001941386.1	7.00E-13	73154	5.00E-43
Contig22633	366	41	No hit			No hit	
Contig22182	1413	38	Predicted protein (*Laccaria bicolor*)	XP_001881269.1	2.00E-63	34172	7.00E-78
Contig00191	850	37	No hit			No hit	
Contig18344	1278	36	No hit			72270	1.00E-24
Contig06220	1362	34	Protein disulphide isomerase (*Laccaria bicolor*)	XP_001888384.1	2.00E-36	117675	2.00E-59
Contig19568	1331	34	Unknown (*Populus trichocarpa* × *Populus deltoides*)	ABK96247.1	2.00E-10	77357	5.00E-24
Contig01984	1291	32	RE56164p (*Drosophila melanogaster*)	AAN71436.1	4.00E-05	No hit	
Contig18144	108	32	No hit			No hit	
Contig19643	880	31	No hit			No hit	
Contig04210	1667	30	Glycoside hydrolase family 61 protein (*Laccaria bicolor*)	XP_001883194.1	9.00E-13	78608	3.00E-68
Contig03336	1183	30	Expansin family protein (*Laccaria bicolor*)	XP_001877082.1	9.00E-22	71932	3.00E-47
Contig18608	1065	30	No hit			104797	3.00E-16

*Length of contigs in nucleotides and number of reads in the assembled contigs.

†BLASTX searches were carried out against the National Center for Biotechnology Information (NCBI) nonredundant nucleotide (nr) database.

‡BLASTX searches were carried out against predicted secreted proteins (SP) in the *M. larici-populina* (Mlp) Joint Genome Institute (JGI) genome sequence.

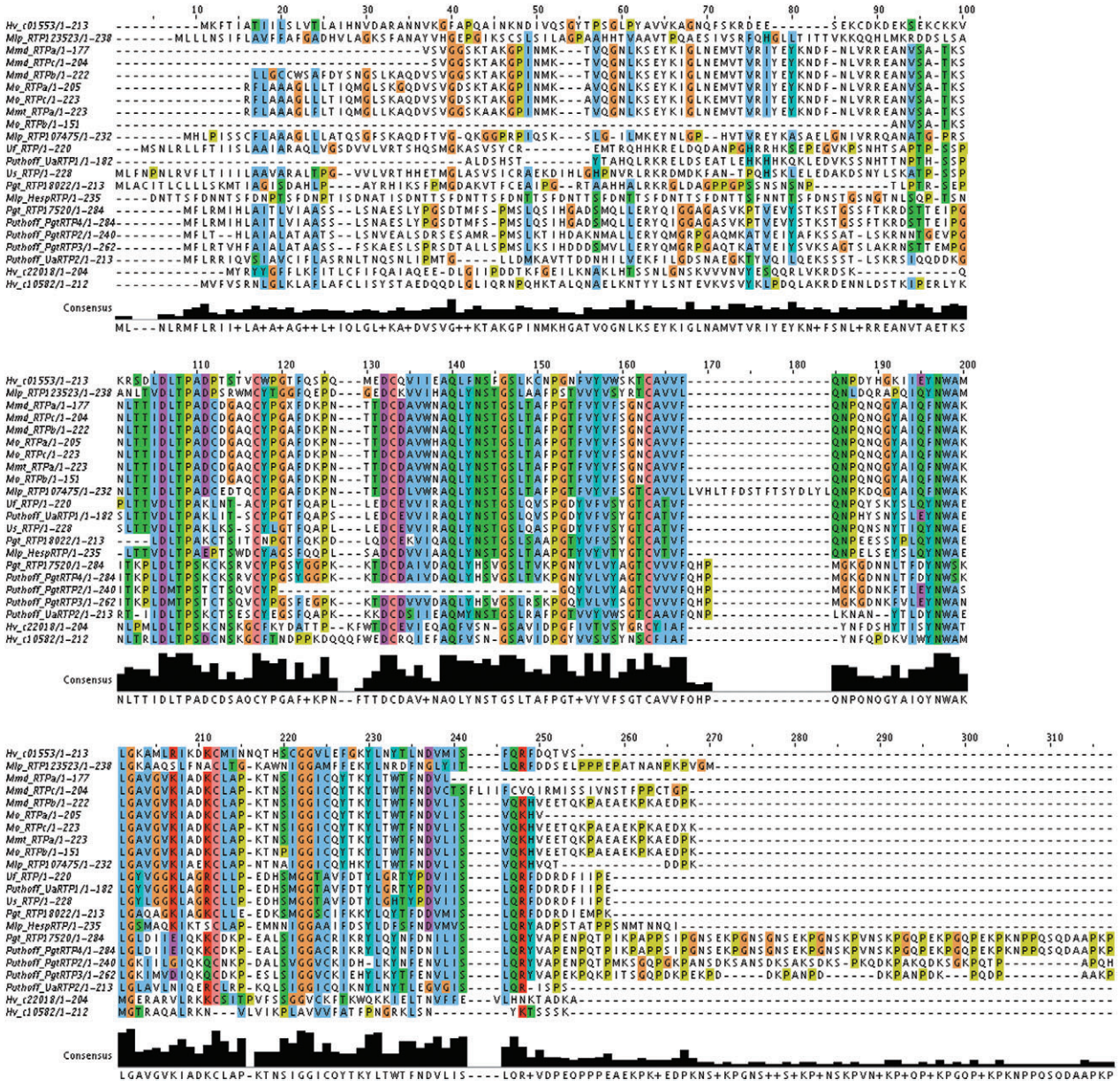


Fig. 3 CLUSTALW alignment of rust proteins homologous to the *Uromyces fabae* rust transferred protein 1 (RTP1). Proteins were retrieved from the literature and international databases. Hv, *Hemileia vastatrix*; Mlp, *Melampsora larici-populina*; Mmd, *Melampsora medusae* f.sp. *deltoidae*; Mmt, *Melampsora medusae* f.sp. *tremuloidae*; Mo, *Melampsora occidentalis*; Pgt, *Puccinia graminis* f.sp. *tritici*; Ua, *Uromyces appendiculatus*; Uf, *Uromyces fabae*; Us, *Uromyces striatus*. Sequences retrieved from Puthoff *et al*. (2008) are indicated. Mmd, Mmt and Mo RTP1-like proteins were predicted from expressed sequence tags (ESTs) and lacked the N-terminal region. Mlp and Pgt sequences were retrieved in the genome sequences available at the Joint Genome Institute and the Broad Institute, respectively. Mlp_HespRTP1 is a fusion protein with homology to a haustorially expressed secreted protein (HESP) in the N-terminal region, and only the C-terminal region presenting homology to RTP1 was used here to perform the alignment.

454-pyrosequencing. Microscopic evaluation of fungal differentiation indicated that, at 18 hpi, the fungus had germinated and differentiated appressoria. At 48 hpi, most infection sites were at the penetration hyphae stage, with the first haustoria formed in stomatal cells. At 7 dpi, all structures, including haustoria in the host mesophyll, had differentiated and no major changes in relative proportions were recorded from this point onwards; at 21 dpi, the leaves were heavily colonized. RT-qPCR expression profiling was conducted in two independent time-course replicates.

Fold-change expression levels measured for the 22 genes at each time point, compared with the average expression level

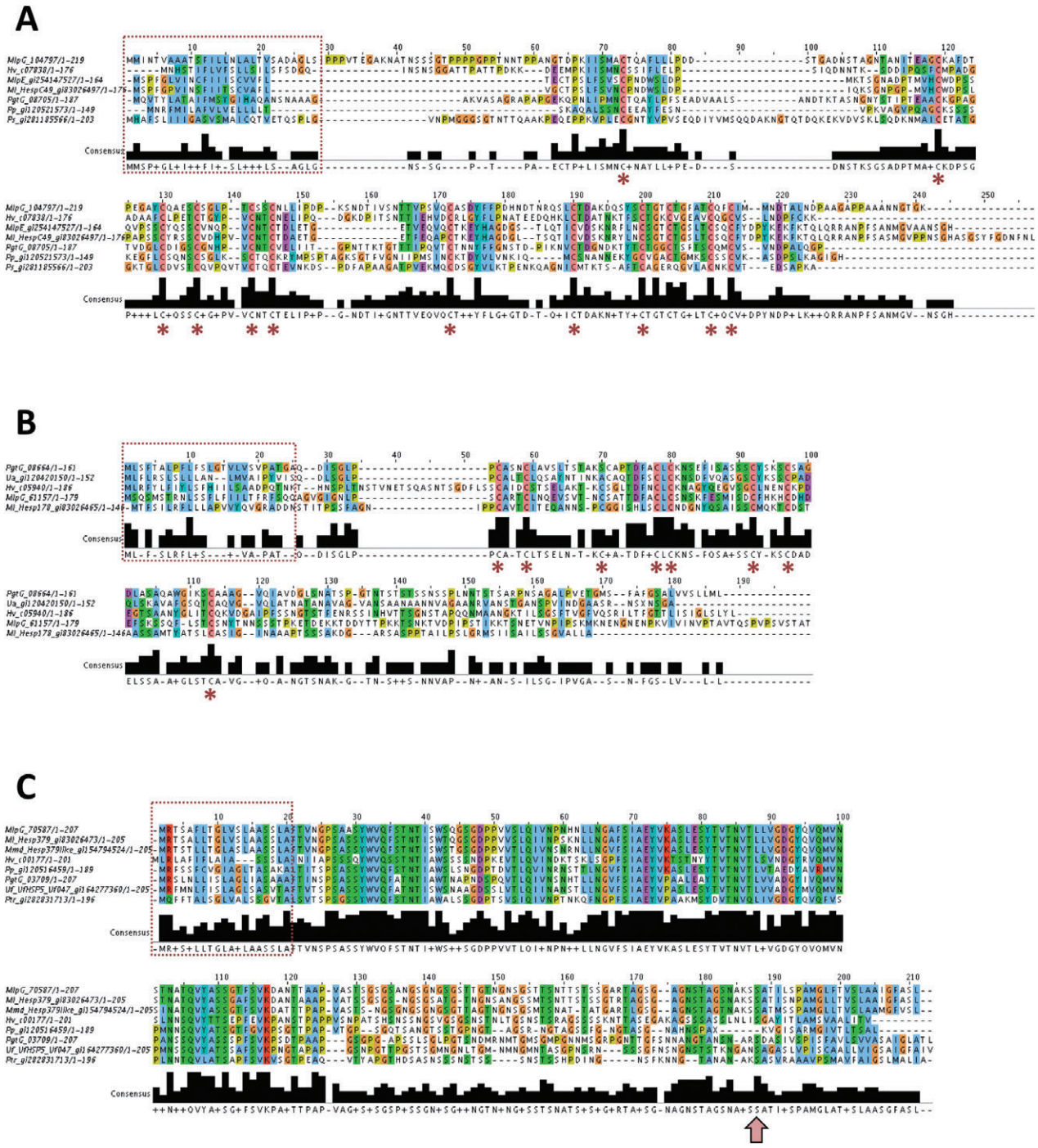


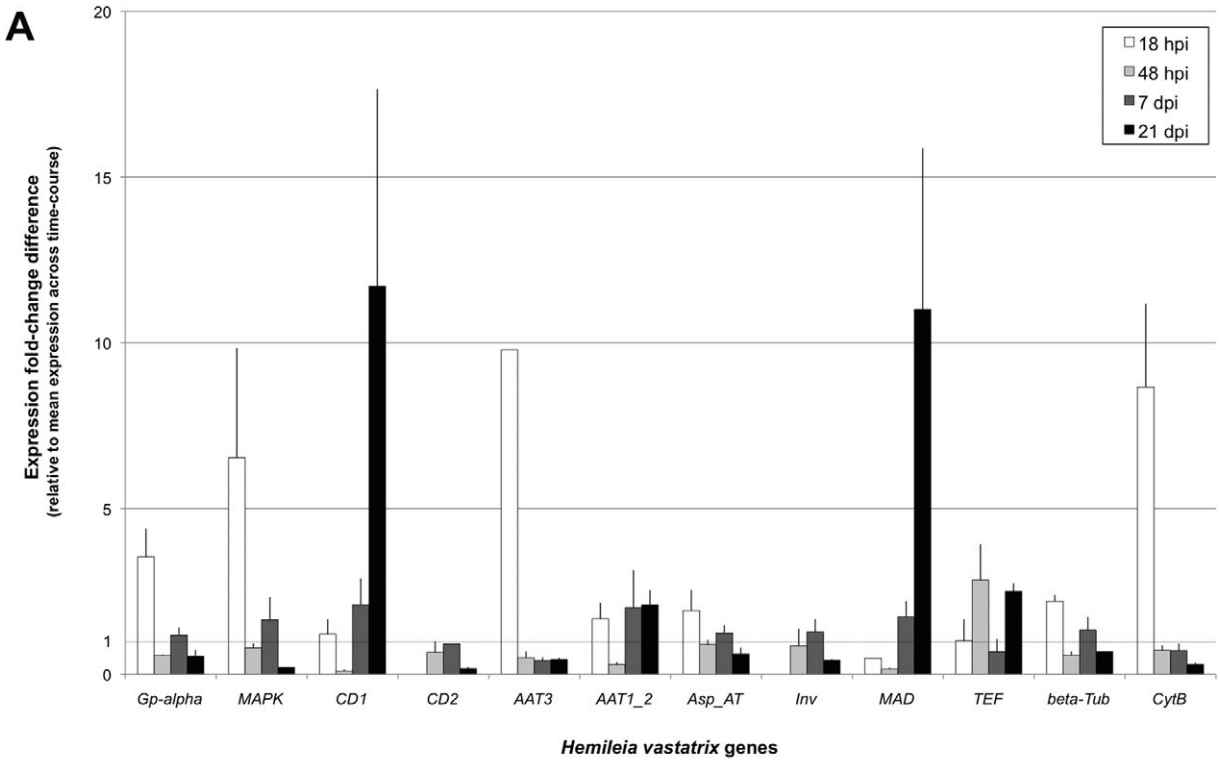
Fig. 4 CLUSTALW alignments of conserved rust secreted protein sequences presenting homology with *Melampsora lini* haustorially expressed secreted proteins (HESPs). (A) Rust homologues of HESP-C49. (B) Rust homologues of HESP-178. (C) Rust homologues of HESP-379. Hv, *Hemileia vastatrix*; Ml, *Melampsora lini*; Mlp, *Melampsora larici-populina*; Mmd, *Melampsora medusae* f.sp. *deltoidae*; Pgt, *Puccinia graminis* f.sp. *tritici*; Pp, *Phakopsora pachyrhizi*, Ps, *Puccinia striiformis*; Ptr, *Puccinia triticina*; Ua, *Uromyces appendiculatus*; Uf, *Uromyces fabae*. MlpG and PgtG indicate that sequences were retrieved from the genome sequences, whereas MlpE indicates sequences deduced from expressed sequence tags (ESTs). Dotted rectangles indicate the N-terminal regions predicted to contain the signal peptides for secretion. In (A) and (B), conserved cysteines are indicated by red stars below the consensus sequence. In (C), the red arrow indicates the glycosylphosphatidylinositol (GPI) anchor location determined by the PredGPI prediction server for all HESP-379 homologues except Pp_gi120516459.

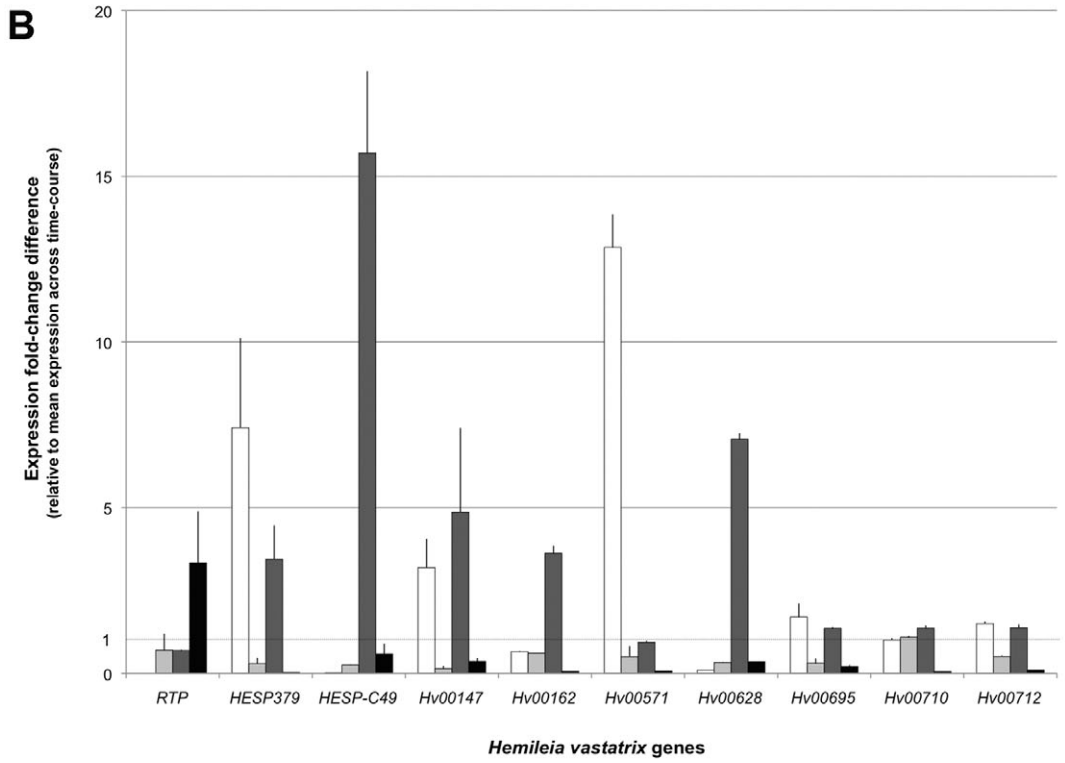
measured along the time course, are presented in Fig. 5. Half of the *H. vastatrix* genes showed almost no or moderate regulation of transcript accumulation during plant infection. By contrast, other genes presented a strong induction of expression at 18 hpi (*Gp-α*, *MAPK*, *CytB*, *AAT3*, *HESP-379* and *Hv00571*), 7 dpi (*HESP-379*, *Hv00147*, *Hv00162*, *Hv00628*) or 21 dpi (*CD1*, *MAD* and *RTP*). Interestingly, distinct expression profiles were obtained for the *CD1* and *CD2* genes, as well as for the *AAT1_2* and *AAT3* genes, indicating a specificity of expression during the interaction with the plant host for these rust multigene families. Amplification cycle values (Cq) measured at 21 dpi for *H. vastatrix* genes were consistent with the number of reads in the corresponding 454-contigs, most abundant contigs presenting lower Cq values than genes with lower expression levels (Fig. S4, see Supporting Information).

DISCUSSION

For years, avirulence genes mediating resistance in host plants have been searched for in fungal pathogens through direct genetic approaches (Stergiopoulos and de Wit, 2009). The concept of effector triggered immunity (ETI) (Jones and Dangl, 2006) has led plant pathologists to enlarge the search to so-called effectors and not only avirulence genes (Dodds and Rathjen, 2010). With the huge collection of genomic data now available for the research community, which is increasing each year, the description of plant pathogen secretomes and of putative virulence effectors detected among secreted proteins has dramatically increased (Lebrun and Kamoun, 2010), and shows evidence that fungal genomes encode a large reservoir of effector-like proteins (Choi *et al*., 2010; Soanes *et al*., 2008). In this article, we report the use of the Roche 454-pyrosequencing technology to establish a repertoire of *H. vastatrix* transcripts expressed in coffee leaves to search for candidate virulence effectors of the coffee orange rust fungus. More than 350 000 reads were produced and assembled into 22 774 contigs from plant or fungal transcripts. A common strategy for the identification of transcript origin in EST sequencing projects based on mixed plant tissues and fungal infection structures usually involves the performance of homology searches using the BLASTX algorithm against the nr databases at GenBank, although such an approach has clear limitations because of the over-representation of plant sequences relative to fungal ones (e.g. 12 578 963 Eudicotyledon ESTs and 629 036 Basidiomycota ESTs in dbEST in August 2010). Predictive methods, based on GC percentage, codon usage or di-, tri- or hexa-nucleotide frequencies in ESTs, have also been used, and programs have been designed to help define the plant or fungal origin of ESTs in plant–pathogen interaction studies (Bowen *et al*., 2009; Emmersen *et al*., 2007). In the absence of the availability of extensive *Coffea* and *Hemileia* genomic data, an original predictive approach was selected that combined the two methods described above. The prediction of plant or fungal origin of rust-infected coffee leaf 454-contigs was completed by systematic comparison of the sequences with genomic data available for three dicots and three basidiomycete species, as well as plant and fungal sequences deposited in GenBank, and *Coffea* and Pucciniales ESTs deposited in dbEST. Finally, the combined prediction approach resulted in about 30% fungal contigs and 61% plant contigs, and the remaining sequences were considered as not determined. *Coffea arabica* is an allotetraploid species that contains two subgenomes related to the *Coffea canephora* and *Coffea eugenioides* species (Lashermes *et al*., 1999; Petitot *et al*., 2008). As of August 2010, 43 619 *C. arabica* ESTs were available in public EST databases. As reported, the *C. arabica* genome is complex and no sequencing project is yet planned for this economically important crop species. The discovery of around 14 000 novel *C. arabica* transcripts provides new opportunities to decipher the allotetraploid coffee genome and may enhance our knowledge of the genetics and physiology of this crop species. When the *C. canephora* genome is made publicly available (http://www.coffeegenome.org/), it will be possible to compare the contigs produced in the present study to update the accuracy of contig selection and to aid in the annotation of gene prediction. Although fungal contigs represented 30% of the contigs, the proportion of 454-sequences was much less in the rust contigs than in the coffee contigs (i.e. 20% of the total number of reads). A high proportion of *H. vastatrix* proteins

Fig. 5 Expression profiles for 22 *Hemileia vastatrix* transcripts during the time course of infection of *Coffea arabica* var. H147/1 leaves by *H. vastatrix* strain 178a at 18 and 48 h post-inoculation (hpi) and 7 and 21 days post-inoculation (dpi). (A) *Hemileia vastatrix* genes with known homology in databases and encoding an heterotrimeric G-protein α subunit (*Gp-alpha*), a mitogen-activated protein kinase (*MAPK*), two chitin deacetylases (*CD1* and *CD2*), two amino acid transporters (*AAT1_2* and *AAT3*), an aspartate amino-transferase (*Asp_AT*), an invertase (*Inv*), a mannitol dehydrogenase (*MAD*), a translation elongation factor (*TEF*), a β-tubulin (*beta-Tub*) and a cytochrome B (*CytB*). (B) Ten *H. vastatrix* genes of no known function and showing homology to known rust effectors (rust transferred protein RTP; haustorially expressed secreted proteins HESP379 and HESP-C49), or showing no homology in international databases and representing new rust genes (*Hv00147*, *Hv00162*, *Hv00571*, *Hv00628*, *Hv00695*, *Hv00710*, *Hv00712*). Transcript levels were assessed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) using three *H. vastatrix* reference genes (cytochrome oxidase subunit III, contig19515; ribosomal protein 40S, contig01333; hypothetical protein Hv00099, contig21737) and, for each gene, fold-change differences correspond to the expression level measured at a given time point relative to the mean level calculated across the time course (note the one-fold line indicated). Data were obtained from two independent time-course replicates.





without any known function was found, which highlights the need for the annotation of fungal genome sequences.

Among the most important cellular categories represented in the fungal contigs were translational- and post-translational-related functions, as well as energy production and conversion. By 21 dpi, a large number of *H. vastatrix* haustoria and infection hyphae had formed in *Coffea*-infected coffee leaves (Silva *et al*., 2006). Rust haustorial structures are involved in the derivation of nutrients from the host tissues to the fungus (Voegele *et al*., 2009). Several transcripts corresponded to transporters previously reported to be specifically or predominantly expressed in haustoria of the bean rust fungus (Struck *et al*., 2002; Voegele *et al*., 2001, 2009), as well as at the uredinial stage of the poplar rust (Hacquard *et al*., 2010). The gene expression patterns observed here indicate that the fungal structures are highly active at this stage of the interaction with the plant host. Carbohydrate, lipid and amino acid transport and metabolism categories were also supported by a large number of rust contigs, supportive of metabolite fluxes between *H. vastatrix* and its host. Expression profiling of selected *H. vastatrix* genes mostly confirmed the expression assessed by read abundance in 454-contigs (Fig. S4). Distinct patterns of rust gene expression were observed during the time course of infection of coffee leaves (Fig. 5), indicative of a highly dynamic transcription activity during plant colonization and biotrophy. Signalling genes encoding a Gp-α and a MAPK showed peak expression at early stages when the fungus had differentiated appressoria, as reported previously for other pathogenic fungi (Li *et al*., 2007), indicating that these genes might also be required for appressorium differentiation in the coffee rust fungus. Interestingly, two genes encoding amino acid transporters homologous to *U. fabae* transporter genes (Voegele *et al*., 2009) showed distinct patterns of expression, *AAT3* being highly expressed at 18 hpi, and *AAT1_2* being slightly induced at 18 hpi and also at 7 and 21 dpi. The latter gene was also expressed in *U. fabae* haustoria (Hahn *et al*., 1997) and at late stages of infection of poplar leaves by *M. larici-populina* (Hacquard *et al*., 2010). The profiles observed here suggest the specific expression of transporter genes at different stages of host colonization. The *CD1* gene showed a strong peak of expression at 21 dpi, consistent with the late expression of a chitin deacetylase encoding gene of the poplar leaf rust fungus in uredinia (Hacquard *et al*., 2010). Strong *MAD* transcript accumulation in haustoria and protein localization in haustoria and uredospores *in planta* have been reported for the bean rust fungus (Voegele *et al*., 2005), and mannitol was described as a possible major source of storage. The strong expression of the *H. vastatrix MAD* gene homologue at 21 dpi suggests that mannitol might play a general role in rust biology, and it remains to be determined whether this polyol serves as a source of storage or is used by the fungus during sporulation.

Using programs predictive for signal peptide secretion signatures with stringent criteria, 382 transcripts encoding putative secreted proteins were identified within the 6763 fungal contigs (Table 3), consisting of 5.6% of the rust contigs in the dataset. Comparison between the genomes of the poplar rust and the wheat stem rust fungi revealed that they encode important reservoirs of secreted proteins, several hundreds being specific to each rust species (Choi *et al*., 2010; S. Duplessis *et al*., unpublished data). The present analysis identified several *H. vastatrix* transcripts homologous to other rust transcripts previously described in rust fungi and related to the biotrophic phase. In contrast, 154 contigs did not show any homology in databases, and may be *H. vastatrix*-specific secreted proteins. Interestingly, 3.9% of the total number of fungal contigs were classified into the intracellular trafficking, secretion and vesicular transport category, among the most important cellular class expressed in the coffee rust fungus, which is supportive of an important secretion activity at this stage of the compatible coffee–rust interaction. Previously, consistent amounts of secreted proteins have been reported from *in planta*-expressed rust transcripts, particularly from purified haustoria (Catanzariti *et al*., 2006; Jakupovic *et al*., 2005; Link and Voegele, 2008; Puthoff *et al*., 2008). More recently, a combined laser capture microdissection (LCM) and transcriptome analysis of poplar rust uredinia has shown that the fungal cells present in uredinia subjacent zones (i.e. haustoria and infection hyphae in the plant palisade mesophyll) are also marked by the accumulation of secreted proteins coding transcripts (Hacquard *et al*., 2010). This suggests that effector-like rust secreted proteins are not only produced at early stages of infection in haustoria, but all along the infection process up to uredospore formation. The important proportion of *H. vastatrix* transcripts homologous to rust secreted proteins detected at this late stage of coffee leaf infection is in accordance with these observations.

In *U. fabae*, the translocation of the secreted protein RTP1 to the plant nucleus has been shown by immunolocalization (Kemen *et al*., 2005), indicating that rust proteins may not be secreted only to the apoplast, but could target plant subcellular locations. Interestingly, this RTP1 protein, although of unknown function, has homologues in *H. vastatrix* and in other rusts, and these are organized in multigene families. The N-terminal part of the protein is quite diverse, whereas the C-terminal part of the protein is relatively well conserved among Pucciniales (Fig. 3), suggesting that it may be related to the function of the protein in the host plant. This result is in accordance with the data of Puthoff *et al*. (2008), who reported that rust putative effectors may be conserved throughout the order Pucciniales, although differences in protein composition could reflect diversification driven by host–rust co-evolution. An *H. vastatrix RTP* gene was strongly induced at a late stage of infection, and not expressed at 18 hpi (Fig. 5), which is consistent with the expression profiles

reported previously in *U. fabae* (Kemen *et al*., 2005) and *U. appendiculatus* (Puthoff *et al*., 2008). The identification of the putative function of RTP will help to clarify this domain organization in the different rust species, and will be of interest to determine whether rust effectors interact with host plant proteins through their C-terminal part, as reported for oomycete effectors (Schornack *et al*., 2009). With regard to *H. vastatrix* transcript homologues of *M. lini* HESPs, although poorly conserved at the global sequence level, a high conservation of Cys positions was found between homologues of HESP-C49 and the CFEM-containing HESP-178 (Fig. 4). This result suggests that these fungal proteins may share a role in pathogenicity or in enabling infection of the host plant. It remains to be determined whether the role of these conserved Cys residues is in coping with the apoplastic environment enriched in plant proteases or in a more particular function during the interaction with the host targets. By contrast, HESP-379 homologues are highly conserved in their N-terminal region and contain a predicted GPI anchor in the C-terminal region, indicating that this protein might reside at the exterior of the rust cell attached to the plasma membrane. A HESP-379 homologue from *U. fabae* (Uf_HSP5_Uf047_gi164277360 in Fig. 4C) has been described previously in the haustorial secretome of the bean rust fungus (Link and Voegele, 2008), suggesting that this conserved rust protein could be secreted by *H. vastatrix* haustoria during the interaction with *C. arabica*. Distinct patterns of expression were observed for *H. vastatrix HESP-379* and *HESP-C49* genes, with expression peaks at 18 hpi and 7 dpi, respectively (Fig. 5). Interestingly, several new rust genes of unknown function, including the *Hv00628* gene encoding a secreted protein, were highly induced at 18 hpi or 7 dpi, highlighting the dynamic expression of rust genes during host infection (Fig. 5). Strikingly, although most selected genes assessed by RT-qPCR were primarily identified at 21 dpi, only four showed their highest expression level at this stage compared with earlier time points. This result indicates the need to dissect the plant infection process for a better understanding of the rust genetic programs expressed *in planta*.

With the use of a new-generation sequencing technology in a nonmodel rust species, we have been able to report the expression of novel genetic determinants, particularly rust candidate effectors. However, it is not possible here to detail in which rust subcompartment the detected transcripts were expressed. The LCM technique, which allows the isolation of subareas in tissues, represents an interesting methodology to dissect rust biology at the uredinial stage. Recently, LCM has been applied to separate *Phakopsora pachyrhizi* uredospores from emerging uredinia (Tremblay *et al*., 2009), and has helped to identify 925 ESTs expressed *in planta* in this structure. Similarly, LCM has been used to dissect different fungal structures at the uredinial stage of poplar leaf infection by the rust fungus *M. larici-populina* and has revealed, by custom oligoarray-transcriptome profiling, that distinct genetic programs related to biotrophy and sporulation are expressed by the rust structures (Hacquard *et al*., 2010). Combining such an LCM approach with new-generation sequencing will probably provide major improvements in our understanding of rust biology.

The data reported here are new and represent the first steps towards a molecular understanding of the pathogen by providing a global view of the *H. vastatrix* and *C. arabica* interacting transcriptomes. Using data collected at an advanced stage of the fungal developmental cycle in the leaf, we have detected novel coffee and fungal gene transcripts and predicted putative rust pathogenicity genes. Approximately 60% of the *H. vastatrix* contigs did not map to any previously annotated protein coding genes and represent novel and interesting sequences. Currently, more than 45 *H. vastatrix* races have been described (Rodrigues *et al*., 1975; Várzea and Marques, 2005). By identifying *H. vastatrix* genes linked to pathogenicity, a genetic survey of the different races could be undertaken to determine the level of variability and the genetic relationships among the virulent populations. Insights into allelic diversity in *H. vastatrix* effector gene loci will reveal the mechanisms underlying the dynamics of adaptation to plant defences in populations of the rust fungus. In addition, gene data may help in the design of molecular diagnostic tools to identify *H. vastatrix* races.

EXPERIMENTAL PROCEDURES

Biological material and infection procedures

Coffea arabica CIFC H147/1 plants (carrying the resistance factors S_H2, S_H3, S_H4 and S_H5) were grown in the glasshouse in 70% relative humidity at 25 °C and under 12 h light. Plants were inoculated with *H. vastatrix* as described in Silva *et al*. (1999) with fresh uredospores of *H. vastatrix* isolate 178a (bearing the virulence factors v_2, v_3, v_4 and v_5), eliciting a compatible interaction. In this H147/1 × 178a pathosystem, sporulation occurred at 25–26 dpi (data not shown). For 454-pyrosequencing, leaves were collected at 21 dpi, ahead of sporulation, frozen by immersion in liquid nitrogen and stored at −80 °C until RNA extraction. For the time-course infection experiment, leaves of *C. arabica* H147/1 inoculated with *H. vastatrix* isolate 178a were collected at different time points after inoculation (18 hpi, 48 hpi, 7 dpi and 21 dpi) in order to represent the progression of infection and host tissue colonization. Successful uredospore germination, appressoria formation and fungal growth in host tissues were confirmed by light microscopy after staining with cotton blue lactophenol. This was performed either by the observation of leaf surface moulds (18 hpi) or of leaf sections, as described

previously (Silva *et al*., 1999). Material was immediately frozen in liquid nitrogen and stored at −80 °C.

RNA extraction and cDNA synthesis

Total RNAs were extracted from coffee leaves using the RNeasy Plant Minikit (Qiagen, Courtaboeuf, France) completed by a DNase treatment. One microgram of total RNA was used to produce cDNA using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). RT and PCRs were carried out with PrimeScript™ Reverse Transcriptase (Takara Bio, Shiga, Japan) and Advantage® 2 Polymerase Mix (Clontech), respectively. The cDNA purification steps were performed using a Qiaquick PCR Purification Kit (Qiagen).

Pyrosequencing and assembly of 454-reads

Sequences were generated from 5 μg of full-length cDNA using the 454-GS-FLEX Titanium pyrosequencing technology on a Roche 454 Titanium sequencer (single read strategy) at the Genoscope (Centre National de Séquençage, Evry, France; http://www.genoscope.cns.fr/spip/) employing a half-plate and following standard procedures recommended by Roche. The assembly of the 352 146 pyrosequencing reads was performed at the Genoscope using the Roche Newbler assembler (software release 2.0.00.22) following recommended parameters provided by Roche. Raw 454-sequences are available upon request. The 22 774 assembled CaHv (*C. arabica* leaves infected by the rust fungus *H. vastatrix*) contig sequences are provided in Data S1. The relative abundance of transcripts was estimated according to Vega-Arreguín *et al*. (2009) with the following index: $Ra = N/L$, where Ra is the relative abundance, N is the number of 454-sequences per contig and L is the length of the assembled contig.

PCR and RT-qPCR

In order to determine the origin of the 22 selected 454-contigs, PCR amplifications were carried out on an MyCycler thermocycler (Bio-Rad, Hercules, CA, USA), using specific primers designed after the 454-contig sequences, with Dream Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), following the manufacturer's protocol for a total of 40 cycles. *Hemileia vastatrix* appressoria cDNA, rust-infected *C. arabica* leaf cDNA and healthy *C. arabica* leaf cDNA were used as template DNA, and control amplifications with no template DNA were also performed. Amplification products were separated by electrophoresis on 3% agarose gels using a Gene Ruler Low Range marker (MBI Fermentas).

In order to assess transcript levels by RT-qPCR, the specific primers of the 22 *H. vastatrix* genes were used and expression analysis was conducted along a *H. vastatrix* differentiation/infection time course at 18 and 48 hpi and at 7 and 21 dpi in two independent time-course replicates. First-strand cDNAs were synthesized from 1 μg of total RNA in a final volume of 20 μL using an Omniscript Reverse Transcriptase Kit (Qiagen) and oligo(dT)18 primer (MBI Fermentas) following the manufacturer's instructions. RT-qPCR was performed on a MJ Mini thermocycler (Bio-Rad) equipped with a MiniOpticon Real Time PCR System (Bio-Rad) using EvaGreen Supermix (Bio-Rad), following the manufacturer's recommendations. Three *H. vastatrix* genes validated as reference genes (A. Vieira *et al*., unpublished results) were used to normalize *in planta* expression (cytochrome oxidase subunit III, contig19515; ribosomal protein 40S, contig01333; hypothetical protein Hv00099, contig 21737). For each gene, fold-change expression levels were obtained using the relative expression formula of Pfaffl (2001), where the average expression level across all sampled time points was used as control.

Sequence databases used and EST annotation

In the absence of extensive genomic data for both *C. arabica* and *H. vastatrix* (43 619 ESTs and no EST, respectively, in dbEST as of August 2010), sequence homology searches were performed against public databases [nr and Swiss-Prot databases at NCBI (http://www.ncbi.nlm.nih.gov/); PHI-base v3.2], a reference database (Winnenburg *et al*., 2008; http://www.phi-base.org/) and three distinct database sets generated for this study. The first set (database I) was composed of the three dicot genomic databases (*A. thaliana*; *Populus trichocarpa* cv. Nisqually; *Vitis vinifera*, http://www.phytozome.net/) and three basidiomycete genomic databases [*L. bicolor* and *M. larici-populina* at the Joint Genome Institute (JGI), http://www.jgi.doe.gov/; *P. graminis* f.sp. *tritici* at the Broad Institute, http://www.broadinstitute.org/]. The second set (database II) included only *Coffea* spp. and Pucciniales spp. ESTs built from sequences retrieved from dbEST at GenBank. Reference ESTs were cleaned on the basis of systematic searches against nr DB using the BLASTN algorithm (E-value $\leq 10^{-10}$) and by removing hits corresponding to plasmid vectors, bacteria and animal sequences, and fungal sequences in the *Coffea* EST set or plant sequences in the Pucciniales EST set. ESTs with no homology in nr were not considered. Totals of 41 241 and 23 168 reliable *Coffea* reference ESTs and Pucciniales reference ESTs, respectively, were retained in database II. Finally, the third set (database III) was narrowed to Pucciniales sequences, with a total of 155 280 transcribed sequences retrieved from the NCBI database (May 2010), and classified into 10 groups based on the corresponding rust species of origin (i.e. 203 *P. graminis* f.sp. *tritici* ESTs; six *Puccinia coronata* var. *lolii* ESTs; 2850 *Puccinia striiformis* f.sp. *tritici* ESTs; 47 466 *P. triticina* ESTs; 27 *M. lini* cDNA and DNA sequences; 49 017 *M. larici-populina* ESTs; 34 394 *Phakopsora pachyrhizi* ESTs; 601 *U. fabae* ESTs; 19 480 *Uromyces appendiculatus* ESTs; and 1236 *Cronartium quercuum*

f.sp. *fusiforme* ESTs). Homology searches were performed using BLAST algorithms (Altschul *et al*., 1997) against genome sequences and predicted transcripts (BLASTN), deduced protein sequences (BLASTX) and rust ESTs (TBLASTX) with a cut-off criterion (*E*-value $< 10^{-5}$). For each search against a given database, only the best BLAST hit was considered and the corresponding *E*-value, scores and sequence match length were extracted using local scripts. The assignment of 454-contig sequences into KOG functional categories was obtained using Reverse psi-BLAST (RPSBLAST; Altschul *et al*., 1997) against the KOG database (Tatusov, 2003).

Identification of plant and fungal transcripts from 454-derived contigs

Contigs of assembled 454-reads were further considered for the analysis, whereas singletons were not included. Two main approaches were used to help distinguish fungal sequences from plant host sequences, and are described here. In total, six predictions were made (Table 2; Figs S1 and S2) and the number of plant (nP) or fungus (nF) predictions for each contig was used to distinguish between contigs. Contigs that were only predicted as plant (nP > 2 and nF = 0) or fungus (nF > 2 and nP = 0) were considered as plant or fungus, respectively. When contigs had been alternatively attributed to plant or fungal origins, the following codes were used: likely plant (nP $\geq$ 4 and nF = 2; or nP > 2 and nF = 1; or nP = 1 and nF = 0); dubious plant (nP > nF and nF > 1); likely fungus (nF $\geq$ 4 and nP = 2; or nF > 2 and nP = 1; or nF = 1 and nP = 0); dubious fungus (nF > nP and nP > 1); not resolved (nP = nF). Finally, when none of the six predictions identified a sequence as P or F, the sequence was registered as 'not determined'.

Species-specific trinucleotide frequencies were considered using the EST3 program from MIPS trained with reference sequences (Emmersen *et al*., 2007). A first training set (intrinsic training set) was constructed on the basis of homology searches against database II for the contigs obtained after the assembly of the 454-reads (Fig. S1). A second EST3 training set (extrinsic training set of 300 plant and 300 fungal sequences) was built from database II sequences, based on the *Coffea* and Pucciniales ESTs homologies to the nr database (i.e. highest BLAST scores only to plant or fungal sequences in nr). The EST3 program trained with the extrinsic training set predicted a number of plant and fungal sequences from the contigs obtained after the assembly of the 454-reads. From this prediction, three sets of 300 predicted *Coffea* and 300 predicted *Hemileia* contigs were selected at random to build three intrinsic training sets to improve EST3 prediction. The EST3 program trained with these random sets of intrinsic *Coffea*/*Hemileia* sequences predicted different numbers of plant and fungal sequences. The contigs that were always predicted as plant or fungus by EST3 using either intrinsic or extrinsic training sequences were considered as tentative plant and tentative fungal contigs, respectively.

Homology searches (BLASTN and BLASTX) were performed against the public databases or the database sets generated for this study (Fig. S2). For the homology searches performed against the public databases and database II, contigs were predicted to be of plant or fungal origin, respectively, based on the best BLAST hit. For database I searches, average BLAST scores were calculated for positive hits against dicots and basidiomycetes, and were then subtracted (i.e. mean plant scores subtracted by mean fungal scores). If the value obtained was higher than 100 (arbitrary cut-off), the contig was considered to be of plant origin, whereas a value below −100 (arbitrary cut-off) was considered to be of fungal origin. Genes of known plant or fungal origin were manually inspected and the {−100;100} cut-off values were validated for origin detection (data not shown). The contigs obtaining a value between 100 and −100 were systematically manually inspected considering the length and percentage of identity between the query and subject sequences to determine the eventual origin. When all BLAST result parameters were too close to clearly define the origin, the contigs were considered as 'not determined' (nd). All prediction and homology search results for the 22 774 CaHv contigs are detailed in Table S1.

Bioinformatic analysis of fungal transcripts

Contigs assigned to the rust fungus *H. vastatrix* were systematically compared with database III and PHI-base using the BLASTX algorithm. Comparisons were also extended to the proteomes of ascomycete fungi, including hemibiotroph and necrotroph plant pathogens, on the MycorWeb portal (data not shown; http://mycor.nancy.inra.fr/IMGC/TuberGenome/blast.php). In order to identify fungal sequences encoding secreted proteins, open reading frames (ORFs) were predicted with the translation tool FrameFinder of the ESTate package (http://www.ebi.ac.uk/~guy/estate/) using a codon usage table defined with the TBLASTX alignment results of 2461 reliable *H. vastatrix* contigs (F_contigs, Table S1, column L). The accuracy of the ORF prediction was assessed by comparing the results of homology searches against the nr and Swiss-Prot databases with the protein sequence translated from the predicted ORF (BLASTP) and the initial best BLAST hit obtained with the contig nucleotide sequence (BLASTX). ORFs below 25 amino acids were not considered. A secretome bioinformatic pipeline previously used to detect secreted proteins in fungal genome sequences (Martin *et al*., 2008; 2010) was employed to define a tentative set of secreted proteins encoded by *H. vastatrix* transcripts. Briefly, the pipeline is composed of the SignalP v3.0 and the TargetP 1.1 programs (Bendtsen *et al*., 2004; Emanuelsson *et al*., 2000) for signal peptide and/or subcellular

localization predictions, and the TMHMM v2.0 program (Krogh *et al*., 2001) to predict transmembrane (TM) helices. Stringent criteria were used to consider a protein as secreted: (i) secretion prediction by TargetP; (ii) all SignalP scores (i.e. Smean, Smax, D_score, hmm_pred) positive; (iii) no TM helix or, in the case of prediction of a single TM, overlap with the signal peptide. Deduced protein sequences and secretion prediction results are detailed in Table S2. Alignments of RTP and HESP rust homologues were performed with the CLUSTALW2 program available at the European Bioinformatics Institute website (Larkin *et al*., 2007) (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The identification of GPI anchor sites in sequences of HESP-379 homologues was performed using the PredGPI prediction server (Pierleoni *et al*., 2008) (http://gpcr2.biocomp.unibo.it/predgpi/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1 Complete list of 22 774 CaHv contigs assembled from 454-GS-FLEX Titanium reads sequenced from rust-infected *Coffea* leaf tissues in FASTA format. The contig sequences are tagged according to their origin (*Coffea* or *Hemileia*). Contigs not resolved or not attributed are also included in the list and tagged accordingly. Before each sequence, the FASTA definition line also includes the length of contig (length=) and the number of reads in the contig (numreads=).

Fig. S1 Bioinformatic procedure used to determine the origin of the sequences produced by the 454-GS-FLEX Titanium pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* (CaHv 454) using the difference in the trinucleotide frequencies of plant and fungal sequences with the EST3 program at MIPS (Emmersen *et al*., 2007; http://mips.helmholtz-muenchen.de/proj/est3/).

Fig. S2 Bioinformatic procedure used to determine the origin of sequences produced by the 454-GS-FLEX Titanium pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* (CaHv 454) using BLAST searches in selected *Coffea* spp. and Pucciniales expressed sequence tags (ESTs), nonredundant nucleotide (nr) and Swiss-Prot databases at the National Center for Biotechnology Information (NCBI) and genomic resources (genome sequences, predicted gene models, deduced proteins). Final CaHv 454 contig prediction is based on both EST3 prediction (detailed in Fig. S1) and BLAST searches.

Fig. S3 Confirmation of fungal origin by polymerase chain reaction (PCR) amplification of fungal and plant cDNA samples for 22 selected contigs predicted to be of fungal origin by the bioinformatic approach. Electrophoretic separation on 3% agarose gels of PCR amplification products after 40 cycles is presented. Gel lanes are as follows: HvA, *Hemileia vastatrix* appressoria cDNA; iL, infected leaf cDNA; hL, healthy leaf cDNA; N, no-template control; M, gene ruler low-range marker (MBI Fermentas; detailed next to *Hv00695* gel). The following is the list of genes assessed with their corresponding contigID and expected amplicon size: *Hv00162* (contigID_02863, 178 bp); *Hv00571* (contigID_15588, 192 bp); *Hv00710* (contigID_14082, 209 bp); *Hv00712* (contigID_06198, 162 bp); *HESP379* (contigID_00177, 197 bp); *HESP-C49* (contigID_07838, 176 bp); *AAT1_2* (contigID_15333, 220 bp); *AAT3* (contigID_01489, 246 bp); *MAD* (contigID_01480, 94 bp); *RTP* (contigID_05622, 75 bp); *Inv* (contigID_17450, 76 bp); Hv00628 (contigID_02411, 169 bp); *Gp-alpha* (contigID_00973, 146 bp); *TEF* (contigID_00300, 128 bp); *CytB* (contigID_19719, 120 bp); *beta-Tub* (contigID_06023, 196 bp); *Hv00695* (contigID_12408, 215 bp); *Asp_AT* (contigID_05141, 72 bp); *CD1* (contigID_14071, 350 bp); *CD2* (contigID_07966, 160 bp); *MAPK* (contigID_11618, 204 bp); *Hv00147* (contigID_04496, 222 bp).

Fig. S4 Relationships between the number of reads in 454-contigs and the average Cq value measured at 21 days post-inoculation for 22 genes assessed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in this study and three reference fungal genes. Lower Cq values indicate higher transcript levels.

Table S1 Complete list of homologies found for the 22 774 454-contigs of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* (CaHv contigs) against the various databases tested in this study. Columns A–C detail the contig_ID, length of contig and number of reads in the contig; columns D–I detail the results of the distinct predictions carried out (F, fungus; P, plant; nd, not determined); columns J–L detail the number of P and F predictions and the final prediction made for contig origin (F, likely F and dubious F for fungal sequences and P, likely P and dubious P for plant sequences); columns N–T detail the results of the different EST3 predictions; columns V–AE detail the BLAST hits obtained against selected *Coffea* spp. and Pucciniales expressed sequence tags (ESTs); columns AG–CZ detail the BLAST hits obtained against three selected dicots and three selected basidiomycete genomes (BLASTN), predicted gene models (BLASTN) and deduced proteins (BLASTX); columns DB–DN detail the BLAST hits obtained against the nonredundant (nr) and Swiss-Prot databases (National Center for Biotechnology Information, NCBI). Lines 2–6764 correspond to contigs attributed to *H. vastatrix*; lines 6767–20 717 correspond to contigs attributed to *C. arabica*; lines 20 720–22 077 correspond to contigs not resolved; lines 22 078–22 779 correspond to contigs not attributed. The different categories of contigs are sorted by read number.

Table S2 Prediction of *in planta*-expressed secreted proteins in *Hemileia vastatrix* 454-contigs (Hv contigs). Protein sequences

were predicted from Hv contigs using framefinder (columns D–I), and secretion prediction (column J) was based on predictions of secretion and transmembrane domains with SignalP, TargetP and TMHMM (columns K–AB). Columns AC–AS detail the best hits against euKaryotic Orthologous Group (KOG) categories (RPS-BLAST against KOG database), *Melampsora larici-populina* secreted proteins [BLASTX against predicted proteins, Joint Genome Institute genome website], nonredundant and Swiss-Prot protein databases (BLASTP; National Center for Biotechnology Information) with corresponding accession numbers, *E*-values and scores. Hv contigs in the table are sorted by secretion prediction (column J) and by contig read number (column C).

Table S3 *Hemileia vastatrix* 454-contig homologies with Pucciniales expressed sequence tags (ESTs) and the Pathogen–Host Interaction database (PHI-base). TBLASTX searches were performed against nucleotide sequences of PHI-base (v3.2) and ESTs of the following Pucciniales species retrieved from dbEST at the National Center for Biotechnology Information: *Cronartium quercuum* f.sp. *fusiforme*, *Melampsora larici-populina*, *Melampsora lini*, *Phakopsora pachyrhizi*, *Puccinia triticina*, *Puccinia striiformis*, *Puccinia graminis*, *Puccinia coronata* var. *lolii*, *Uromyces appendiculatus*, *Uromyces fabae*.

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