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By Artur Alves

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A multi-omics analysis of the grapevine pathogen *Lasiodiplodia theobromae* reveals that temperature affects the expression of virulence- and pathogenicity-related genes

Carina Félix[1], Rodrigo Meneses[1,2], Micael F. M. Gonçalves[1], Laurentijn Tilleman[3], Ana S. Duarte[1], Jesus V. Jorrín-Novo[4], Yves Van de Peer[2,5,6], Dieter Deforce[3], Filip Van Nieuwerburgh[3], Ana C. Esteves[1, a], Artur Alves[1,*]

[1]Department of Biology, CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

[3]Laboratory of Pharmaceutical Biotechnology, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium

[4]Agroforestry and Plant Biochemistry, Proteomics and Systems Biology, Department of Biochemistry and Molecular Biology, Universidad de Córdoba, Córdoba, Spain

[5] Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium

[6] Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria 0028, South Africa

[a]current address: Portuguese Catholic University, Health Science Institute-Viseu, Estrada da Circunvalação, 3504-505 Viseu

*Corresponding author:

Artur Alves

Telephone: (+351) 234370776

Fax: (+351) 234 372 587

e-mail address: artur.alves@ua.pt

Abstract

Lasiodiplodia theobromae (Botryosphaeriaceae, Ascomycota) is a plant pathogen and human opportunist whose pathogenicity is modulated by temperature. There is little information regarding the molecular effects of temperature on *L. theobromae*, so we used a multi-omics approach to understand how temperature affects the molecular mechanisms of pathogenicity.

The genome (Illumina HiSeq) of *L. theobromae* LA-SOL3 was sequenced and annotated. Furthermore, the transcriptome (Illumina TruSeq) and proteome (Orbitrap LC-MS/MS) of LA-SOL3 grown at 25 °C and 37 °C were analysed.

Proteins related to pathogenicity (plant cell wall degradation, toxin synthesis, mitogen-activated kinases pathway and proteins involved in the velvet complex) were more abundant when the fungus grew at 25 °C. At 37 °C, proteins related with pathogenicity were less abundant than at 25 °C, while proteins related with cell wall organization were more abundant. On the other hand, virulence factors involved in human pathogenesis, such as the SSD1 virulence protein, were expressed only at 37 °C.

Taken together, our results showed that this species presents a typical phytopathogenic molecular profile that is compatible with a hemibiotrophic lifestyle. We showed that *L. theobromae* is equipped with the pathogenesis toolbox that enables it not only to infect plants but also animals.

Introduction

Lasiodiplodia theobromae (Pat.) Griffon & Maubl. (Botryosphaeriaceae, Ascomycota), an emergent grapevine trunk disease agent, is frequently the most commonly found species in vineyards, as well as the most aggressive, especially in warmer climates[1, 2]. This pathogen has been associated to almost 500 plant hosts and a large number of diseases, especially in crops[3]. In humans, *L. theobromae* has also been associated to several diseases with different levels of severity, from ocular infections to human death[4, 5, 6, 7,8].

Changes in the climate, namely increasing temperature, can influence the behaviour of pathogens[9]. Nonetheless, little effort has been directed to identify the impact of these alterations on microbial pathogen/host interactions, that ultimately can lead changes in virulence[10, 11, 12].

It has been suggested that the expression of virulence factors in *L. theobromae* can be modulated by temperature13, 14 (Félix et al. 2016, Yan et al. 2017). Úrbez-Torres et al. (2011)[1] and later Yan et al. (2017)[14] showed that the larger lesions caused by *L. theobromae* on grapevines were observed when the plants were grown at 35 °C. However, it is unclear if this results from increased virulence of the pathogen or increased susceptibility of the host due to heat stress effect.

An approach integrating multiple omics technologies can be used to understand the functional principles and the dynamics of cellular systems[15, 16]. In the last years, some studies in species of the family Botryosphaeriaceae, including *L. theobromae*, revealed important findings during *in vitro* short term heat stress response. Some of these findings were the expansion of gene families associated with cell wall degradation, nutrient uptake, secondary metabolism and membrane transport, genes in carbohydrate-binding modules, lysine motif domain and the glycosyl hydrolase families induced by

high temperature[14] or up-regulated genes encoding for enzymes, with the ability to degrade salicylic acid and plant phenylpropanoid precursors[17].

Sequencing and annotation of a pathogen's genome opens the possibility of performing multi-omics approaches and, identify gene and gene products that are affected by temperature. The first genome of *L. theobromae* was also recently released[14]. However, none of the studies used a multi-omics integrated approach for a more detailed comprehensive characterization of the species.

The aim of this study was to understand how *in vitro* growth temperature affects the expression of pathogenicity and virulence-related genes in a strain of *L. theobromae* isolated from grapevine. For this, we integrated genome sequencing and RNA-seq with proteomics data to study the molecular basis of *L. theobromae* pathogenicity at different temperatures.

Results

Genome sequencing, assembly and annotation

The genome of *L. theobromae* LA-SOL3 was sequenced into 38.8 million matched paired-end reads by Illumina sequencing (Table 1). Assembly led to a genome size of 43.9 Mb (43,925,482 bp) with approximately 214 x coverage, divided into 413 scaffolds with minimum size of 2 Mb, a total of 79 gaps and 2,368 Ns (undetermined nucleotides), as well as an overall G+C content of 54.75 %, much lower than the described earlier for *L. theobromae* CSS-01s[14]. The N50 value was approximately 249 kb. RepeatMasker analysis, coupled with RepeatModeler and RepeatProteinMask, discovered an overall repeat content of 2.21 % of the genome. The genomic features of *L. theobromae* LA-SOL3 are shown in table 1.

BRAKER software predicted 12,785 genes, with an average length of 1,610 bp, a gene density of 291 genes/Mb and a total of 46.8 % of the genome covered by protein-coding genes. There is an average relation of 1 mRNA per gene, with an average of 2.8 exons and 1.8 introns per mRNA, per gene.

Lasiodiplodia theobromae LA-SOL3 genome encodes 667 extracellular proteins. From these, 7.3 % (335) of all enzymes encoded by the genome are enzymes. While the most represented enzymes have oxidoreductase (1299 in total, 46 secreted), transferase (962 in total, 7 secreted) or hydrolase (1486 in total) functions, secreted enzymes are mainly hydrolases (237). Other enzymes were also identified, such as, lyases (291 in total, 28 secreted), isomerases (119 in total, 10 secreted) and ligases (192 in total), although some of the predicted genes were classified in more than one enzyme category.

Quite a number of genes related to pathogenicity and virulence were identified. Among them are oxidoreductases and CAZymes. Oxidoreductases are known for being involved in plant pathogenesis in processes such as the degradation of lignin and the detoxification of reactive oxygen species produced by the host[18], while CAZymes participate in the colonization and infection of plant pathogenic fungi, disassembling the plant cell wall[19,14]. In total, *L. theobromae* LA-SOL3 genome encodes for 1299 oxidoreductases (46 are predicted to be extracellular) and 789 CAZymes (272 are predicted to be extracellular). The largest number of CAZymes are glycoside hydrolases, accounting for 317 proteins (127 secreted, Figure 1).

Among oxiredutases, forty genes encode peroxidases, with a total of 69 functions (Table S1). The number of functions was higher than the number of proteins, since 14 genes were annotated with two or more peroxidase types.

Secondary gene clusters are responsible for the synthesis of relevant secondary metabolites, that are believed to be involved in the development of certain disease

symptoms and, in some cases, in host death. *Lasiodiplodia theobromae* LA-SOL3 genome encodes 52 secondary metabolite gene clusters – per type: 8 terpenes, 17 t1pks (type 1 polyketide synthases), 11 nrps (nonribosomal peptide-synthetase), 6 t1pks-nrps and 10 "other". Of these, 7 clusters were found to have known homology to other described clusters: NRPS – chaetocin (13 %) and hexadehydro-astechrome (HAS) (25%); terpene – PR toxin (50 %); T1PKS – brefeldin (20%), emericellin (50%) and lasiodiplodin (71%) and T1PKS-NRPS – fusaridone A (12 %).

In pathogenic fungi, cytochrome P450 enzymes are usually related with their defence against toxic substances produced by the hosts. In fact, it is known that P450 is involved in the production of mycotoxins (*e.g.* aflatoxins), and gibberellins[20]. A total of 43 genes were annotated as coding for fungal cytochrome P450, within 23 different P450 families (Table S2).

Several studies suggest that transporters play a crucial role in pathogenicity of fungi, contributing to different functions such as drugs secretion or the transport of molecules involved in appressoria formation[14]. In the genome of *L. theobromae* LA-SOL3, 1957 genes code for transporters (Table S3).

Heat shock proteins (HSP) are known to be involved in several common biological activities, such as transcription, translation, protein folding, and aggregation and disaggregation of protein, protecting proteins from damages caused by different types of biotic and abiotic stresses[21]. Different families of HSP known to be specifically involved in responses to heat stress were identified in the genome of *L. theobromae* (Table S4).

Transcriptome and proteome of *L. theobromae* LA-SOL3 at two temperatures

Temperature deeply influenced the growth (Figure S1) and both the transcriptome and proteome (Figures S2 and S3).

A total of 1,580 genes were determined to be differentially expressed, whereof 1,059 genes were annotated, 849 genes were down-regulated and 731 genes were up-regulated at 37 °C (Figure S2).

According to GO enrichment analysis (Table S9), heat stress leads to an increase of the number of transcripts related to primary metabolism, plant cell wall degradation and pathogenesis, and to a decrease of transcripts related with stress response, carbohydrate metabolism and catabolism and transport functions (Figure 2).

A total of 269 proteins were identified in the secretome of the fungus grown at 25 °C and only 15 proteins in the secretome of the fungus grown at 37 °C (15 of which were less abundant than at 25 °C). Regarding the intracellular proteome, a significantly higher number of proteins were identified: 1,312 proteins for 25 °C and 662 for 37 °C (17 more abundant and 241 less abundant than at 25 °C). In both cases, secretome and intracellular proteome, the number of identified proteins was higher when the fungus was grown at optimal temperature (25 °C) (Figure 3).

When *L. theobromae* LA-SOL3 grows at 37 °C, there is a considerable increase of extracellular proteins related with cell wall organization (CWO), primary metabolism (PM) and stress response (SR). Proteins directly related to pathogenesis (P) were either not detected – in the extracellular compartment (Tables S10, S11, Figure 4) – or less abundant intracellularly (Figure 5). On the other hand, proteins related with protein metabolism were more abundant in the intracellular proteome at 37 °C. The analysis of the biological of differentially expressed transcripts and proteins was performed (Figure 6), shows a similar trend of abundance.

Some transcripts and proteins previously implicated in for pathogenesis, were identified in this study: transcripts/proteins involved in salicylic acid degradation, members of the Velvet complex and mitogen-activated protein kinases (MAPKs). A virulence factor, the virulence protein SSD1 (Q5AK62) was exclusively identified in the intracellular proteome, at 37 ºC, while the phytotoxin Snodprot1 (O74238) was identified only in the secretome of LA-SOL3 at 25 ºC.

Discussion

The genome of *L. theobromae* LA-SOL3 was compared with that of *L. theobromae* CSS-01s (Table 2), a strain that was also isolated from grapevine.

The genome of CSS-01s sequenced by Yan et al. (2017)[14] encodes 937 secreted proteins, accounting for about 7.26 % of their predicted proteome size. In the genome of LA-SOL3, about 5.30 % of LA-SOL3 deduced proteome size corresponds to secreted proteins. This difference may be related to use of the algorithm FunSec. Funsec integrates the prediction of signal peptides and transmembrane regions, subcellular localization and endoplasmic reticulum motifs. Therefore, FunSec performs a very stringent analysis leading to a reduced number of false positives.

The analysis of the genome *L. theobromae* LA-SOL3 is in accordance to the transcripts and proteins identified at 25 and 37 ºC: all the functions identified in the genome were also identified in the transcriptome and proteome, although temperature favoured some functions towards others.

From the total amount of genes, 192 genes encode pathogenesis related genes (GO:0009405). From these 119 were differentially transcribed at 25 ºC and 37 ºC, and 143 were detected as proteins differentially expressed at 25 and 37ºC (including proteins only detected at one of the temperatures).

At 25 °C (Figure 5), *L. theobromae* LA-SOL3 expresses a large amount of extracellular proteins involved in plant cell wall degradation (18.4 %) and in pathogenesis (25.6 %), while at 37 °C the percentage of proteins involved in pathogenesis decreased (proteins relevant for pathogenesis are described in Tables 3, S9 and S10). In fact, under stress conditions (37 °C) (Figure S1), the energy of the fungus was directed to guarantee the survival of the organism, and to the maintenance of the cell wall integrity.

From those groups of proteins relevant for pathogenesis (Table 3), we highlight the presence of proteins involved in toxins' synthesis and of MAPKs that are more abundant in the proteome of LA-SOL3 grown at 25 °C.

In the case of plant pathogenic fungi, MAPK pathways are known to be required for the full virulence inside the host[18, 22, 23].

We identified genes, transcripts and proteins essential for the cascades of MAPK pheromone pathway (Figure 7) (*STE11-STE7-FUS3*, previously identified in *Saccharomyces cerevisiae*, and *PMK1*, previously identified in *Magnaporthe grisea*). We also identified the MAPK genes involved in protection against high osmolarity (*STE11-HOG1*, previously identified in *S. cerevisiae* and *OSM1* previously identified in *M. grisea*)[22, 24, 25]. The presence of well-represented pathways of MAPKs signalling, that contributes to the successful penetration and dissemination of the fungal pathogen in different hosts, in *L. theobromae* LA-SOL3, helps to explain how this species is able to infect a wide range of plant hosts and to proliferate in different conditions.

Several HSP genes, transcripts and proteins related to heat response, as is the case of the family clpA/clpB (P31540) and family 70 (P15705), were identified (Table S4). The expression of such molecules allows the pathogen to overcome heat stress, since HSP will protect the cells from the possible damages caused by the stress factors[21].

Transcripts and proteins involved in the Velvet complex were identified in the transcriptome and in the intracellular proteome of LA-SOL3 grown at 25 °C. The deletion of one of the proteins of the complex influences the production of specific gene clusters, *e.g.*, the deletion of *LaeA* gene promotes the decrease of virulence factors' production in *Fusarium fujikuroi*[26] (Niehaus et al. 2018). Among these virulence factors are the genes and proteins involved in toxin synthesis, such as in the synthesis of fusaric acid and of fusarin C, identified in the genome, transcriptome and proteome of *L. theobromae*, and in the production of fumonisin (identified in the genome and transcriptome).

The expression of proteins with potential roles in pathogenesis of *L. thebromae* LA-SOL3 (Table 4) was identified.

For all proteins, with the exception of the virulence protein SSD1 and the Snodprot1 protein, that were identified only at protein level, the expression was confirmed at the mRNA level. SSD1 protein was identified only in the proteome of LA-SOL3 grown at 37 °C. This protein is typically involved in human infections caused by fungi, which helps to explain the capacity of *L. theobromae* to infect humans[6].

Previous studies identified jasmonic acid by *L. theobromae* only at 25 ºC[27]. In this study it was possible to identify transcripts (down-regulated) and proteins (only at 25 ºC), related to the synthesis of jasmonic acid [e.g. the enzyme 12-oxophytodienoate reductase 7 (Q6Z965)]. Jasmonic acid is a phytotoxin that can lead to the inhibition of the defense pathway of the plant host, facilitating the infection process[28, 29].

We identified other proteins (detected either at the protein or mRNA level) associated with human infections - allergens, aspartic proteases and proteins involved in toxin synthesis (Tables S9 and S10). These data show that *L. theobroame* has the molecular

246 machinery to colonize and infect humans, and that this machinery is over-expressed
247 when the fungus grows at 37 ºC.
248 Recent studies revealed that genes involved in carbohydrate catabolism, pectin, starch
249 and sucrose metabolism, and pentose and glucuronate interconversion pathways
250 identified in the transcriptome of *L. theobromae,* in the presence of shoots of grapevine,
251 at different temperatures (25 °C and 35 °C), were induced during the infection process[14,
252 17]. Paolinelli and co-authors found that enzymes related with plant cell wall degradation
253 were up-regulated at 35 °C. Also, enzymes with the ability to degrade salicylic acid and
254 phenylpropanoid precursors were up-regulated, suggesting that L-tyrosine catabolism
255 pathway could help evading the host defence response[17]. In our study, we identified
256 (both by transcriptomics and proteomics) gene products involved in the degradation of
257 the plant cell wall up-regulated at 37 °C. We also identified proteins involved in the
258 degradation of salicylic acid, but at 25 °C. Although data of both studies may seem
259 discordant, a direct comparison between our study and that of Paolinelli is not
260 straightforward or accurate: the presence of the host in the study of Paolinelli leads to
261 multiple factors affecting the expression of proteins by *L. theobromae*. In Paolinelli's
262 study not only *L. theobromae* was under stress, but the host, *V. vinifera*, was also under
263 thermal stress.
264 Globally, the results obtained show that *L. theobromae* has not only the ability to
265 behave as a typical phytopathogen, expressing molecules involved in plant cell wall
266 degradation or in the degradation of salicylic acid, but also has the necessary toolbox to
267 infect mammals, expressing proteins typically involved in the infection of humans.
268 Several proteins typically involved in human pathogenesis are expressed only at 37 ºC,
269 suggesting that the pathogenicity of *L. theobromae* is modulated by temperature.

Moreover, the presence of genes and proteins known to be involved not only in necrosis of host tissues (genes/proteins involved in synthesis of toxins or virulence factors as the snodprot protein) but also in mechanisms that allow the pathogen to persist inside a living host (as the degradation of salicylic acid or the expression of the protein SSD1), suggest that this species may have a hemibiotrophic lifestyle.

Having in mind the ongoing climate changes, one may speculate that an increase in temperature although leading to heat stress of *L. theobromae*, may also lead to an increase on the severity of human infections by this opportunist pathogen.

Methods

Fungal strains and culture conditions

The strain used in this study was *L. theobromae* LA-SOL3, isolated from *Vitis vinifera* in Peru. LA-SOL3 was one of the most aggressive strains in artificial inoculations trials of cv. Red Globe plants[2]. The culture was routinely grown on Potato Dextrose Agar (PDA) medium (Merck, Germany) at 25 °C.

For DNA extraction, the mycelium harvested from a culture grown on Potato Dextrose Broth (PDB, Merck, Germany) at 25 °C for 3 days was ground in liquid nitrogen and DNA was extracted according to Möller et al. (1992)[30]. For protein and RNA extraction, LA-SOL3 was inoculated into a 250 mL flask containing 50 mL of PDB and incubated at 25 °C or at 37 °C for 4 days. All assays were performed in triplicate.

DNA extraction, Genome sequencing and assembly

Lasiodiplodia theobromae strain LA-SOL3 was sequenced from 500 ng of genomic DNA by Illumina MiSeq at NXTGNT (Belgium). The DNA was fragmented to 800 bp using Covaris S2 sonication and a sequence library was made using the NEBNext Ultra

295 DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA). During the library
296 preparation, a size selection was performed just before the enrichment PCR using an
297 Invitrogen 1% E-gel to select for fragments between 700 and 1200 bp. Nine PCR cycles
298 were used during the enrichment PCR. The libraries were equimolarly pooled and
299 sequenced on an Illumina MiSeq sequencer, generating ~19E+06 2x300 bp paired-end
300 reads. Base calling and primary quality assessments were performed using Illumina's
301 Basespace genomics cloud computing environment. Reads were trimmed based on
302 quality scores (modified Mott trimming algorithm, threshold=0.05) and reads shorter
303 than 100 nt were discarded. Detected adaptor sequences were also trimmed and reads
304 mapping to the Illumina internal control phage phiX were discarded. Assembly and
305 scaffolding were achieved with CLC Genomics Workbench 9.0.1
306 (https://www.qiagenbioinformatics.com/) *de novo* assembly module with default
307 settings, except for the minimum contig length (2000 nt) or word length (60 nt).
308 Optimal word size was estimated using KmerGenie28[31] prior to the assembly. The
309 primary scaffolds were further refined by means of the SSPACE2 scaffolder. The
310 gapped regions in the re-scaffolded assembly were (partially) closed using GapFiller[32].
311
312 **Gene prediction and annotation**
313 Before gene prediction, the genome was repeat-masked. RepeatModeler (v1.0.8)[33] was
314 used to build repeat libraries for the *L. theobromae* genome, which were then filtered to
315 exclude coding regions and simple repeats. RepeatMasker (v4.0.5) was used to "hard
316 mask" repeats and transposable elements in the genomes, using the curated repeat
317 libraries, replacing repeats and transposable elements by strings of 'N's.
318 Gene prediction was achieved with BRAKER1 (v1) with the "fungus" option. RNA-seq
319 paired-end reads from the GEO study GSE75978[17] were selected (SRR2994047,

SRR2994048, SRR2994053, SEE2994054, SRR2994059, SRR2994060, SRR2994062 and SRR2994063) and used for mapping onto the assembled genome using HISAT2 (v2.0.5)[34] with default settings.

Gene product names were assigned based on predicted protein sequences with BLASTP against the UniProt-KB/SwissProt database (downloaded on March 1st, 2017), with an E-value threshold of 1e-3. Other functional annotations were added with InterProScan (v5.21-60)[35], mapping accessions for InterPro, GO, CDD-3.14 and Pfam-30.0 as well as GO terms. Further functional analyses were carried out as described by Morales-Cruz et al. (2015)[36].

Genome functional analysis

Secreted gene products were predicted with the FunSec pipeline (v1.0) (https://github.com/Lonewolfenrir/FunSec). InterProScan analysis and the Ensemble Enzyme Prediction Pipeline (E2P2) were used to annotate protein sequences. The results were divided into different categories based on their EC numbers. Carbohydrate-degrading enzymes (CAZymes) were predicted with the web-based application dbCAN (HMMs 5.0) with default settings[37]. Fungal peroxidases were predicted with the web-based BLAST application of fPoxDB: Fungal Peroxidase Database[38], against the Whole Predicted Peroxidases database (updated on April 18th, 2012), with an E-value threshold of 1e-5. Gene clusters encoding for secondary metabolites were predicted for the full genomes with the web-based application fungiSMASH (antiSMASH fungal version v4)[39], with added GFF3-formatted files, obtained from gene prediction, and ran with default settings. Cytochrome P450 identification was pursued by BLAST against The Cytochrome P450 Homepage[40] database, with an E-value threshold of 1e-5, against the fungal database (updated on

August 12th, 2009). A BLAST analysis was conducted against the Pathogen Host Interactions database (PHI-base), downloaded on February 2nd, 2017, against the whole proteome and the secreted protein datasets, with an E-value threshold of 1e-5. Transporters were identified with a BLAST analysis against the Transporter Classification Database[41] (Saier et al. 2006), downloaded on January 14th, 2018, using an E-value threshold of 1e-5.

RNA extraction, library preparation and sequencing

The mycelium of each replicate (three replicates per condition) was ground in liquid nitrogen and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma), according to the manufacturer's instructions. Samples were incubated for 15 min with the DNase I digestion set (RNase-Free DNase Set, Qiagen). Integrity and quality analysis were done on a 2100 Bioanalyzer RNA (Agilent Technologies). Afterwards, samples were stored at -80 °C until sequencing library preparation. Illumina mRNA sequencing libraries were made from 500 ng total RNA of each sample using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to manufacturer's protocol. Fourteen PCR cycles were used during the enrichment PCR. The size distribution, purity (absence of free adaptors) and quantity of the resulting libraries were measured using a High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, US). The libraries were equimolarly pooled and sequenced in an Illumina Nextseq 500 high throughput flow cell, generating single-end 75bp reads. After sequencing, the data was demultiplexed using the sample specific nucleotide barcodes. Per sample, on average 38 x 10^6 ± 4 x 10^6 reads were generated. First, these reads were trimmed using cutadapt version 1.11. To remove the "QuantSEQ FWD" adaptor sequence the trimmed reads were mapped to the genome of *L. theobromae* LA-

SOL3 using the STAR aligning software v 2.5.3a[42]. The RSEM software, version v1.2.31, was used to generate count tables.

Proteome analysis

The extracellular fraction of the proteome was obtained with the TCA/Acetone method[43]. The final pellet was resuspended with 200 µL of lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris) and stored at -80 °C.

For the intracellular fraction of the proteome, mycelia were grinded to a fine powder in a mortar in the presence of liquid nitrogen. To the powder, 10 mL of 10 mM potassium-phosphate buffer (pH 7.4) containing 0.07 % DTT and cOmplete™ protease inhibitor cocktail (Roche) was added. All the samples were sonicated [1 min sonication, 2 min pause (3 min of sonication in total) (Branson, Sonifier 250) at 4 °C] and then shaken using an orbital agitator at minimum speed, during 2 h (4 °C). The homogenate was centrifuged (15000 g, 30 min, 4 °C) and the supernatant was collected. Proteins from the extract were precipitated following the same procedure used to extract extracellular proteins.

Protein concentration was determined with the 2-D Quant Kit (GE Healthcare, USA), according to the manufacturer's instructions. All the samples were quantified in triplicate. For both intra and extracellular proteins, 125 µg of sample were diluted (1:1) in loading buffer and analysed by electrophoresis[44]. Lab-casted SDS-PAGE gels ran at 2 W-2 h, 6 W-3 h on 12 % (w/v). The running buffer contained 100 mM Tris, 100 mM Bicine and 0.1 % (w/v) SDS. The samples were denatured at 100 °C for 5 min prior to electrophoresis. Gels were stained with Coomassie Brilliant Blue G-250 (CBB). After staining, gels were scanned on a GS-800 Calibrated Densitometer (Bio-Rad).

After ensuring the quality of the samples, these were electrophoresed and concentrated at the top of the separation gel (visualized as a unique band). This band was manually excised, and protein bands were destained in 200 mM ammonium bicarbonate (AB)/50 % acetonitrile for 15 min and 5 min in 100 % acetonitrile. Proteins were reduced by the addition of 20 mM dithiothreitol in 25 mM AB and incubated for 20 min at 55 °C. The mixture was cooled to room temperature, followed by alkylation of free thiols by the addition of 40 mM iodoacetamide in 25 mM AB in the dark, for 20 min. After that, protein bands were washed twice in 25 mM AB. Proteolytic digestion was performed by adding 12.5 ng.μL^{-1} of trypsin (Promega, Madison, WI) in 25 mM AB, and incubated at 37 °C overnight. Protein digestion was stopped by addition of trifluoroacetic acid (TFA) at 1 % final concentration. Digested samples were dried in a speedvac.

A nano LC analysis was performed using a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) with a C18 75 µm x 50 Acclaim Pepmam column (Thermo Scientific). Previously, the peptide mix was loaded in a 300 µm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in 2 % acetonitrile/0.05 % TFA for 5 min at 5 $\mu L.min^{-1}$. Peptide separation was performed at 40 °C. Mobile phase A was composed by water acidified with 0.1 % formic acid. Mobile phase B was composed by 20 % acetonitrile acidified with 0.1 % formic acid. Samples were separated at 300 $nL.min^{-1}$. Mobile phase B increased from 4 % to 45 % B in 60 min; 45-90 % B in 1 min, followed by a 5 min wash at 90 % B and a 15 min re-equilibration at 4 % B. The total time of chromatography was 85 min.

Eluting peptide cations were converted to gas-phase ions by nano electrospray ionization and analysed in a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific). Mass spectrometer was operated in positive mode. Survey scans of peptide precursors from 400 to 1,500 m/z were performed at 120 K resolution (at 200 m/z) with a 5 × 105

ion count target. Tandem MS was performed by isolation at 1 Th with the quadrupole, CID fragmentation with normalized collision energy of 35, and rapid scan MS analysis in the ion trap. The AGC ion count target was set to 102 and the max injection time was 75 ms. Only those precursors with charge state 2–6 were sampled for MS2. The dynamic exclusion duration was set to 15 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles, meaning the instrument would continuously perform MS2 events until the list of non-excluded precursors diminishes to zero or 3 s, whichever is shorter.

The raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Scientific). MS2 spectra were searched with SEQUEST engine against an in-house built database of proteins deduced from the genomic sequence. Peptides were generated from a tryptic digestion with up to one missed cleavage, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines as variable modifications. Precursor mass tolerance was 10 ppm and product ions were searched at 0 Da tolerance.

Statistical analysis

For transcriptomic analysis, only genes with counts per million (cpm) above 1 in at least 3 samples were retained. EdgeR[45] was used to normalize gene counts and identify the differentially expressed genes (DEGs) using the empirical Bayes quasi-likelihood (QL) F-test, adjusted using the Benjamin-Hochberg False Discovery Rate (FDR) correction to account for multiple comparisons. Fold Change (log2 FC >1 or <-1) and a False Discovery Rate (FDR <0.01) were used as statistical significance indexes.

For proteomics analysis, the peptide spectral matches (PSM) were validated using percolator based on q-values at a 1 % FDR. With Proteome Discoverer software v. 2.1

(Thermo Scientific), peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1 % FDR. The identified proteins were also filtered and considered for analysis only if present in 3 replicates and using at least 3 peptides for identification (Tables S5, S6, S7 and S8). The abundance level was obtained considering the temperature of 25 °C as control ($0.5 \geq FC \geq 2$).

Ethical approval and informed consent

Not applicable.

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596 **Availability of data and material**

597 The datasets generated during and/or analysed during the current study are available in
598 the GenBank repository. Accession Numbers: SUB3910116

Data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CF, ACE and AA planned and designed the research. CF and MFMG performed experiments, RM, LT, YVP, DD, FN analysed genomic and transcriptomics data, CF, JVJ-N and ACE analysed proteomics data, CF and ASD analysed qPCR data. CF, ASD, JVJ-N, ACE and AA wrote the manuscript. All authors read, revised and approved the manuscript.

Acknowledgments

Not applicable.

Figure 1: Predicted CAZymes (A) and predicted secreted CAZymes (B) in the genome of *L. theobromae* LA-SOL3.

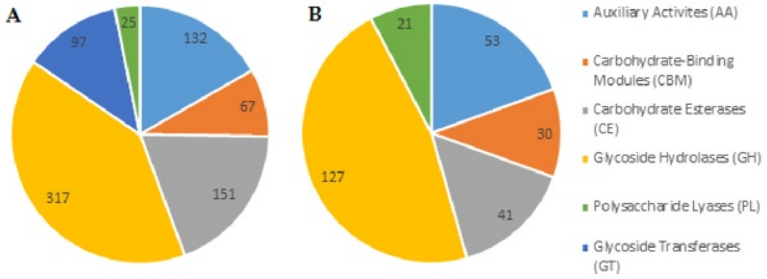

Figure 2: Gene Ontology classification of the transcripts identified in *L. theobromae* LA-SOL3: up and down-regulated transcripts of the fungus grown at 37 °C in comparison with transcripts expressed by the fungus grown at 25 °C. The classification was obtained from the GO (biological process) of each gene product according to the Uniprot database (http://www.uniprot.org/).

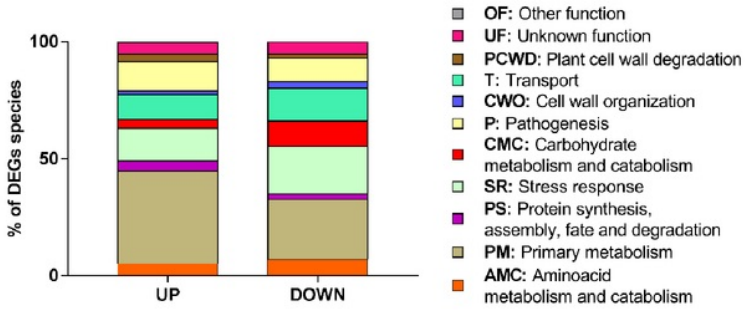

Figure 3: Differential expression of proteins by *L. theobromae* LA-SOL3. Total number of identified extracellular and intracellular proteins (A) and up and down-regulated proteins expressed under heat stress (B). 25 °C corresponds to the control condition.

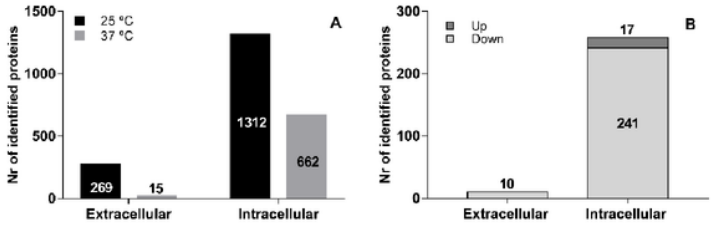

Figure 4: Gene Ontology classification of the extracellular proteins identified in *L. theobromae* LA-SOL3: percentage of distinct species present in extracellular proteins at 25 °C and 37 °C. The classification was obtained from the GO (biological process) of each protein according to the Uniprot database (http://www.uniprot.org/).

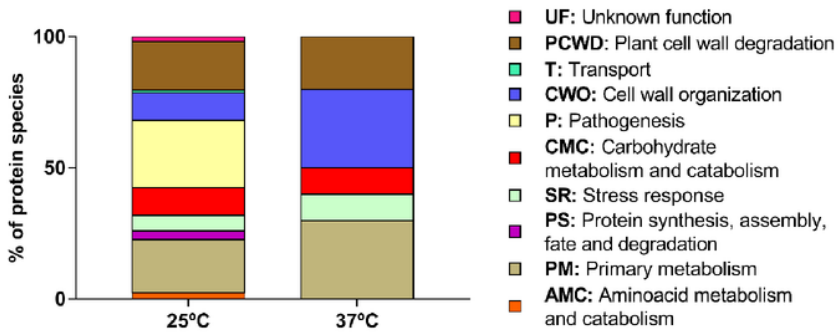

Figure 5: Gene Ontology classification of the intracellular proteins identified in *L. theobromae* LA-SOL3: percentage of distinct species present in extracellular proteins at 25 °C and 37 °C. The classification was obtained from the GO (biological process) of each gene product according to the Uniprot database (http://www.uniprot.org/).

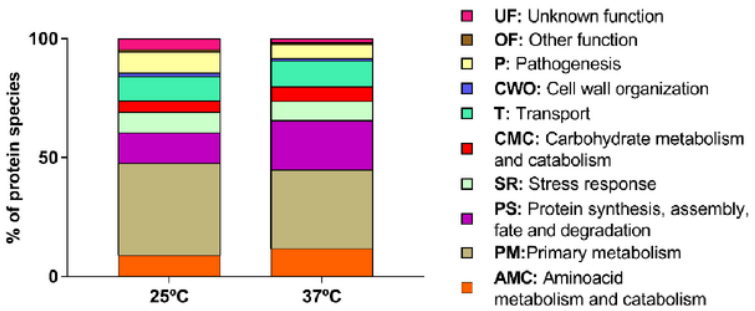

Figure 6: Categorization of *Lasiodiplodia theobromae* differential gene expression and protein levels classified by GO-term (Biological Process) at 25 ºC and 37 ºC. Proteins detected only at one of the tested temperatures were also included in the analysis. Uniprot database was used to access to the GO categories.

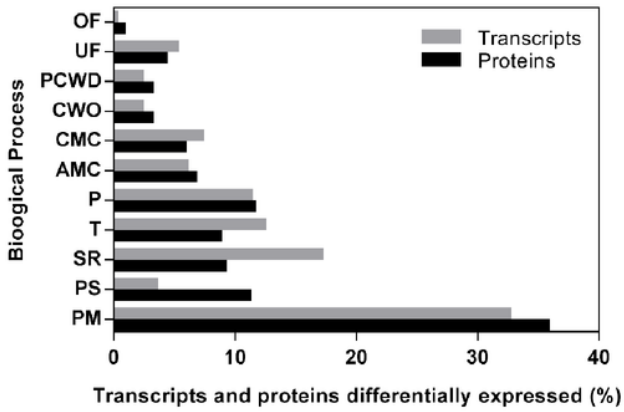

Figure 7: Pathway map assigned for MAPKs signalling. Omics data obtained for LA-SOL3 are highlighted in pink (gene, transcript and protein levels), grey (gene and transcript levels) and blue (transcript level). This figure was adapted from KEGG software, using a reference pathway to construct the map.

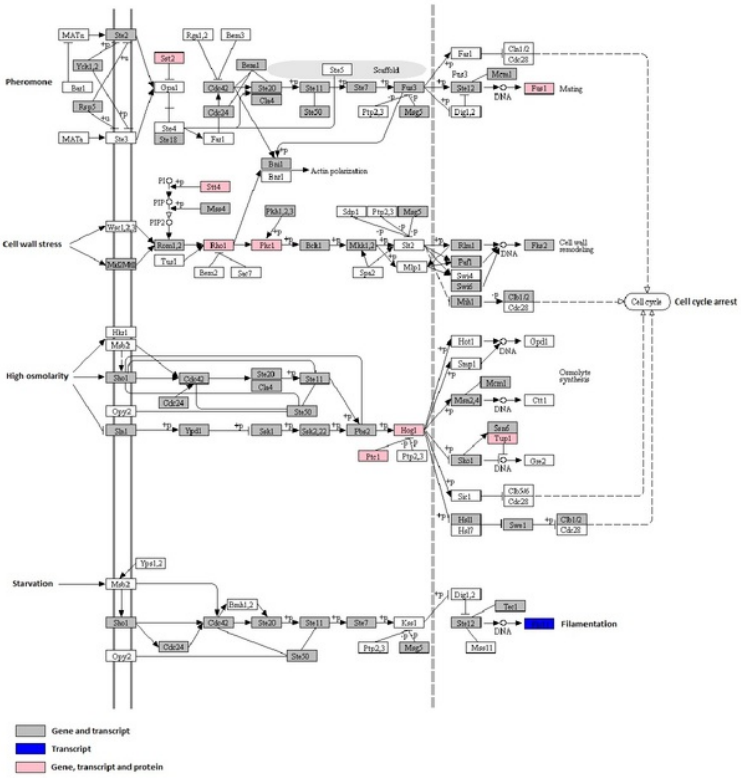

Table 1: - General statistics of *L. theobromae* LA-SOL3 genome assembly, gene prediction and comparison with the available genome of *L. theobromae* CSS-01s (MDYX00000000.1).

Lasiodiplodia theobromae	**LA-SOL3**	**CSS-01s (MDYX00000000.1)**
Size (Mb)	43.9	43.3
Coverage	214 x	90 x
% G+C content	54.75	54.77
% Repeats	2.21	3.02
Number of genes	12785	12902
Average gene length (bp)	1610	1629
% of genome covered by genes	46.8	~48.5
% of genome covered by CDS	42.7	43.6
Gene density (genes/Mb)	291	297
Average exons per mRNA	2.8	2.8
Average exon length (bp)	510	506
Average introns per mRNA	1.8	1.8
Average intron length (bp)	86	88
Average mRNAs per gene	1	n.a.

Table 2: Genes coding for proteins potentially involved in pathogen-plant interaction in the genome of *L. theobromae* LA-SOL3 and comparison with *L. theobromae* CSS-01s (Yan et al., 2017).

	LA-SOL3	**CSS-01s**	**Method**
Total proteins	12785	12902	BRAKER1
Secreted proteins	677	937	FunSec
Enzymes	4579	-	E2P2
Secreted Enzymes	335	-	FunSec + E2P2
CAZymes	789	763	dbCAN
Secreted CAZymes	272	-	FunSec + dbCAN
Metabolic gene clusters	52	58	fungiSMASH
Cytochrome P450s	44	-	The Cytochrome P450 Homepage
Peroxidases	40	-	fPoxDB
Transporters	1957	2419	Transporter Classification Database
Pathogen-Host Interactions	4075	-	PHI-base

678 **Table 3:** Functions identified in the transcriptome and proteome of strain LA-SOL3
679 relevant for pathogenicity.

	Function	Contribution to pathogenicity	Reference
Siderophores	Iron-chelating ligands. Iron transport compounds.	Intracellular iron storage compounds. Suppress the growth of other microorganisms.	Renshaw et al., 2002[46]
Toxins	Polyketides that include a range of compounds as mycotoxins and spore pigments.	Toxic to plants and/or animals. Mode of action extremely variable, depending on the produced compound.	Gaffoor et al., 2005[47] Chauhan et al., 2016[48]
Allergens	Sensitization with extraneous allergens. Fugal allergens are usually proteins, polysaccharides, or glycoproteins.	IgE-mediated hypersensitivity in humans. The major allergic manifestations are asthma, rhinitis, allergic bronchopulmonary mycoses, hypersensitivity pneumonitis.	Fukutomi & Taniguchi, 2015[49]
MAPKs	Mitogen-activated protein kinases that function as key signal transduction components.	Fungal MAPKs help to promote the penetration of host tissues governing appressorium formation and virulence.	Hamel et al., 2012[18] He et al., 2017[23]
HSPs	Involved in several common biological activities, such as transcription, translation, protein folding, and aggregation and disaggregation of proteins.	Involved in stress response. Different families of HSP are expressed depending on stress type, *e.g.*: pH, heat, cold or osmotic stress.	Tiwari et al., 2015[21]
Nudix effectors	Sense and modulate levels of their substrates like nucleotide sugars, deoxyribonucleoside triphosphate and capped mRNAs to maintain proper cellular processes and physiological homeostasis.	Manipulation of host defence systems.	Dong & Wang, 2016[50]
Velvet complex	Regulates fungal development and secondary metabolism.	Promotion of chromatin accessibility and expression of biosynthetic gene clusters involved pathogenicity as mycotoxins, pigments and hormones.	López-Berges et al., 2013[51] Niehaus et al., 2018[26]

680

Table 4: Proteins involved in fungi pathogenesis, identified in the proteome of *L. theobromae* strain LA-SOL3.

Accession number (UniProtKB)	Description	Secretome	Intracellular Proteome	Function	Reference
P14010	4-aminobutyrate aminotransferase	-	25/37 °C	Metabolization of γ-aminobutyric acid to fulfil pathogen nitrogen requirements during infection and manipulates the plant metabolism to maintain/increase the concentration of nitrogen.	Fernandes, 2015[52]
A9MYQ4	Gamma-aminobutyraldehyde dehydrogenase				
Q5AK62	Virulence protein SSD1	-	37 °C	Tolerance of host immune response, allowing the colonization of human tissues by *Candida albicans*.	Gank et al., 2008[53]
C5FBW2 **Q70J59**	Tripeptidyl-peptidase SED2	25 °C	-	Acidification of the microenvironment in the host, facilitating the nutrition and proliferation of the pathogen.	Reichard et al., 2006[54] Félix et al., 2016[13]
D4ALG0	LysM domain-containing protein ARB_05157	25 °C	-	Manipulation of host immune responses to support pathogen colonization.	Kombrink &Thomma, 2013[55] Akcapinar et al., 2015[56]
O74238	Protein SnodProt1	25 °C	-	Cerato-platanin known to manipulate the immune response and cause necrosis in *Ceratocystis fimbriata*. In *Magnaphorte oryzae* a homologue is required for full virulence.	Brown et al., 2011[57]

A multi-omics analysis of the grapevine pathogen *Lasiodiplodia theobromae* reveals that temperature affects the expression of virulence- and pathogenicity-related genes

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