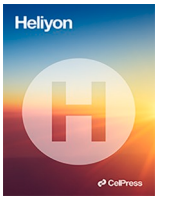
Heliyon 10 (2024) e29124

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Wide transcriptional outlook to uncover *Penicillium expansum* genes underlying fungal incompatible infection

Marta de Ramón-Carbonell [a], Paloma Sánchez-Torres [a,b,*]

[a] *Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, 46113, Moncada, Valencia, Spain*
[b] *Food Biotechnology Department, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Catedrático Agustín Escardino Benlloch 7, 46980, Paterna, Valencia, Spain*

A R T I C L E I N F O

Keywords:
Penicillium expansum
Host specificity
Virulence
Postharvest
SSH

A B S T R A C T

Pathogenesis of *P. expansum* involved different processes and one of them is the recognition between pathogen-host, which in the case of *P. expansum* is preferably pome fruit. In this work, the possible mechanisms connected to host recognition are addressed through the generation of a subtractive library carried out during the incompatible *P. expansum*-orange interaction in the initial stages of infection. The generated library was analyzed by massive sequencing and bioinformatic analysis. Of the identified genes, a total of 24 were selected for subsequent expression analysis by RT-qPCR in two incompatible interaction situations. The characterization of the overexpressed genes revealed the presence of CWDEs, ATPases, aldolases, detoxifying enzymes and virulent determinants that could act as effectors related to fungal virulence independently of the host. However, several identified genes, which could not be associated with the virulence of *P. expansum* under compatible conditions, were related to enzymes to obtain the nutrients necessary for the growth and development of the pathogen under stress conditions through basal metabolism that contributes to expand the range of adaptation of the pathogen to the environment and different hosts.

1. Introduction

Host specificity is defined by the specific recognition of a given host and its colonization. Many plant pathogens show a varied host range, while others are limited to certain species or specific plant families [1,2]. Postharvest diseases in fruits, are mostly produced by *Penicillium* fungal species, including *Penicillium italicum, Penicillium digitatum,* and *Penicillium expansum*. They have a restricted host range, indicating that specific adaptations occur depending on the host. *P. digitatum* and *P. italicum* are generally pathogens of citrus, while *P. expansum* has a wide host range and can colonize various crops, but hardly citrus fruits [3]. The origin of host specificity is believed to be due to the fact that pathogens undergo physiological modifications that allow the growth in the presence of different compounds which are part of the peel of the fruits [4].

The development of host specificity in fungi is driven by a complex mechanism involving several genes and pathways. Some integrative approaches underly the molecular factors that are the key to the progress of host specificity [2]. Among the different *Penicillium* species have been associated to the emergence of protein clusters, genome restructuring, horizontal gene transmission and

* Corresponding author. Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología. 46113, Moncada, Valencia, Spain.
E-mail address: psanchez@iata.csic.es (P. Sánchez-Torres).

https://doi.org/10.1016/j.heliyon.2024.e29124
Received 2 February 2024; Received in revised form 28 March 2024; Accepted 1 April 2024
Available online 4 April 2024

positive selection pressure [2]. The spread of protein families related to fungal pathogenicity would force the speciation of these *Penicillium* species [2]. Knowledge of the processes of both specificity and virulence helps in the search for new alternative control treatments to the fungicides used. Therefore, unraveling the molecular basis of the evolution of host specificity is crucial in emerging plans to reduce losses of economically valuable crops.

Genomes of several *P. expansum* strains are currently available [1,5,6]. Furthermore, Li et al. [7,8] and Ballester et al. [5] addressed the connections between secondary metabolism and infections caused by blue mold. *P. expansum* not only threatens apple production, but also poses food safety concerns by producing a toxin, patulin, which is difficult to remove from contaminated derived food products. Most previous research focused on the challenge of controlling blue mold disease and developing tools to eliminate patulin levels. On the one hand, they have focused on understanding the biosynthesis of patulin and, on the other, on investigating the molecular aspects responsible for the virulence of *P. expansum*, identifying some genes as potential targets for the treatment of blue rot. They highlight the deployment of cell wall degrading enzymes (CWDEs) that allow it to colonize its different hosts and detoxifying enzymes such as glyoxalase [6,9]. Several studies have reported virulence factors studied in different fungi (e.g., *creA, laeA, PacC, Ste12, veA*) [6,10–13], and others have been based on global omics-type studies [14,15].

Despite this, the pathogenicity/virulence along with the development of host specificity in *Penicillium* species remains unclear. In this work, our main objective was to identify the possible mechanisms involved in the host recognition of *P. expansum* and for this we used the subtractive suppression hybridization (SSH) technique that allows generating a cDNA library during the incompatible interaction *P. expansum*-orange. The incompatible interaction forces *P. expansum* to colonize citrus that is not its usual host. Under these conditions, the pathogen is coerced to use an arsenal of resources to adapt to this host. By comparing the genes involved in this process with those induced during infection in pome fruits, we can elucidate some of the mechanisms responsible for host specificity that in turn may be involved in virulence. The wide transcriptional outlook of the identified genes might provide new insights into the complex biological network of *P. expansum*-fruit interactions and the genes responsible for blue decay disease. Furthermore, by exploring host specificity, we could gain insights into its mechanisms and regulation. This fundamental knowledge will allow us to advance, not only in the application of novel and efficient methods in the control of *P. expansum*, but will also deepen our knowledge of fundamental molecular aspects of fungal biology.

2. Materials and methods

2.1. Fruit and microorganisms

Two *Penicillium* strains were used in this study, strain Pex1 (CECT20906) was isolated from Golden rotten apple after storage for several months [5,13] and Pex2 (CECT 2280) provided by the Spanish Type Culture Collection (CECT) with different degree of virulence.

Fungal strains were grown in potato dextrose broth (PDB) or potato dextrose agar (PDA). Cultures were incubated at 25 °C for 1, 2 or 3 days (liquid cultures) depending of the further use or up to 1 week (solid media). Conidia were obtained from 1-week-old PDA plates by scraping them with a sterile spatula, and transferring them to sterile water. Conidia were filtered, and titrated with a hemacytometer and then adjusted to a desire final concentration.

Escherichia coli DH5α was used for proliferation material and plasmid storage. *E. coli* cultures were grown in LB plates or LB broth amended with 100 μg/ml of ampicillin at 37 °C.

'Navel' mature oranges without chemical treatments from IVIA orchards were used for *in vivo* assays and fruit inoculation was done as reported previously [16].

2.2. Molecular manipulations

For SSH experiments total RNA from spores or mycelium of *P. expansum* grown in PDB or water with orange discs in 12 well plates during 24h at 25 °C was obtained from frozen tissue by using Trizol (Invitrogen) following the recommendations of the manufacturer. Total RNA of both *P. expansum* strains grown in flasks with PDB media at 24h, 48h and 72h at 200 rpm and 25 °C was used for expression analysis. Total RNA during at least 3 fruit infection experiments (3 replicas of 5 fruits each) with *P. expansum* was extracted from fruit peel discs of 5 mm diameter from the point of inoculation containing albedo and flavedo at 1-, 2- and 3-days post infection (dpi) as described previously López-Pérez et al. [17]. Poly(A)$^+$ RNA was separated from total RNA using the Dynabeads® mRNA Purification™ kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

2.3. Construction of a subtracted cDNA library

cDNA synthesis and the SSH procedure [18] were conducted using the PCR-Select™ cDNA Subtraction kit (Clontech, Palo Alto, CA, USA) according to the protocol supplied by the manufacturer. *P.expansum* strains were grown in 24 culture plates in presence of 5 mm diameter orange discs containing albedo and flavedo. Control was performed growing fungal strains without orange discs. We used RNA from P.expansum Pex1 at 1 dpi as a 'driver' and RNA from P. expansum Pex1-infecting orange discs tissue at 1 dpi as a 'tester'. One microgram of poly(A)$^+$ RNA from the 'tester' and 'driver' were used for cDNA synthesis. Subtracted cDNA fragments were cloned into pCRII (Invitrogen) vector, and then transformed into E. coli DH5α competent cells by electroporation.

2.4. cDNA sequencing and bioinformatic analysis

The subtracted cDNA library denoted as HSPeS was sequenced using 454-FLX Titanium technology to perform a global transcriptomic analysis of putative fungal host-specificity/virulence genes. Raw sequences obtained were trimmed off vector and adaptor sequences and similarity searches against DNA/protein sequence databases were obtained by means of the BLAST programs [19]. The reference sequences were indexed using "BWA (Burrows-Wheeler Aligner)" [20] against the genome reference sequences corresponding to *Penicillium expansum* (GCF_000769745.1), resulting in a SAM file. SAM file was compressed to BAM format, and the scaffolds were sorted for ease of handling using "SAM" tools [21]. Finally, the BAM file was indexed to allow for visualization and additional analysis in "R Statistical Software" (R Core Team 2021), utilizing the "Rentrez package" [22]. for obtaining the names of indexed genes. The QuickGO tool was used for an initial annotation of Gene Ontology [23].

Heat maps and balloon plot were performed using SRplot package (http://www.bioinformatics.com.cn/srplot).

2.5. Fungal infection

Infection experiments were performed using freshly harvested orange (*Citrus sinensis*) that were injured at four places around the equatorial axis and infected with 10 μL of a conidia suspension adjusted to 10^5 conidia/mL. They were kept at 20 °C and 90% RH. Experiments were performed with 3 replicas of 5 orange fruits (15 in total) and repeated at least 3 times.

2.6. Quantification of relative gene expression by RT-qPCR

Trizol method (Ambion Inc., Austin, USA) was used for RNA extraction from *P. expansum* frozen mycelium after growing in flask with PDB media at 25 °C at 24h, 48h and 72h. Extraction of total RNA from infected samples was processed as reported previously [17].

Contaminating DNA from total RNA was removed with Turbo RNA-free DNAse (Ambion Inc., Austin, TX). Complete elimination of DNA was confirmed by the absence of amplification of a 500 bp fragment of the β-tubulin coding gene of *P. expansum* with oligonucleotides PeTubF and PeTubR (Table S1)

Experimental values obtained were an average of two repetitions of three biological replicates. Different oligos for each EST and genes coding for fungal β-tubulin (qTubF-qTubR), was used as reference gene (Table S1). LightCycler 480 Software, version 1.5 (Roche Diagnostics) was used for cycle quantification. The melting temperature of each primer set allowed the selection of conditions for specific amplification. Relative gene expression was carried out as previously described [24]. PrimeScript™ RT reagent Kit (Takara Bio Inc.) was used for synthesis of the first strand of cDNA in a 20 μl reaction, following the indications of the manufacturer. Quantitative PCR was done as reported before [25]. The software LightCycler 480 SW 1.5 (Roche Diagnostics) was used for cycle point quantification. Primer melting temperature allowed the selection of each primer set for specific amplification. The Relative Gene Expression ('RGE') was carried out as stated before [24].

3. Results

3.1. Sequence examination of the HSPeS library

The subtracted cDNA library HSPeS showed 102284 reads from which 96023 were aligned and only 94967 were assembled. cDNA library contained 1603 singletons, with 133 contigs (Table 1). After sequence assembling, HSPeS is composed by 62 isogroups and 103 isotigs. Sequence processing rendered 154 genes that corresponded to *P. expansum*.

Sequence analysis showed 27 ribosomal proteins, 18 hypothetical proteins without known function and 4 uncharacterized proteins (Table S2). Different genes are distributed among all the sequences that have been associated with proteins categorized with the same function (Table 2).

The most represented EST (PEX1_019060) with 31925 total reads corresponds to the *P. expansum* ATPase, type AAA. Interestingly, within the 20 most abundant genes, two proteins with the same function but that encoded different genes were considered, such as the TIM barrel type aldolase (PEX1_094410 and PEX1_035540) and the helicase (PEX1_018140 and PEX1_045550). Furthermore, it is worth highlighting the presence of 2 chaperones (PEX1_032120 and PEX1_034010), 3 ribosomal proteins and 4 hydrolases

Table 1
Summary of HSPeS cDNA subtracted library.

HSPeS library	
Reads	102284
Aligned Reads	96023
Assembled	94967
Partial	838
Singletons	1603
Contigs	133
Isogroups	62
Isotigs	103

Table 2
List of different genes that encode the same hypothetical function.

Description	Nº of genes
Aldolase-type TIM barrel	3
ATPase	3
Bax inhibitor 1-like protein	2
Chaperonin	2
Glutathione S-transferase	2
Heat shock protein	4
Histone fold	3
Mitochondrial carrier protein	4
Nucleic acid-binding, OB-fold	3

(PEX1_094750, PEX1_055640, PEX1_069360 and PEX1_023750).

3.2. Functional annotation

HSPeS library was analyzed by functional enrichment search of GO (Gene Ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways among differential express genes (DEGs). The enhancement study showed that the most significant upregulated GO terms (Fig. 1) related to biological processes included organic substance biosynthetic, cellular biosynthetic, organ nitrogen-compound metabolic, macromolecule metabolic, cellular nitrogen compound metabolic, protein metabolic, amide metabolic, cellular component organization and transport (Fig. 1A). Within molecular function: heterocyclic compound, nucleoside phosphate, nucleic acid, anion, ribonucleotide cation binding and transmembrane transporter (Fig. 1B). Among cellular components we can stand out intracellular, non-membrane-bounded, membrane-bounded organelle and mitochondrion (Fig. 1C). KEGG pathways revealed the metabolic pathways as the most represented followed by carbohydrate metabolism and biosynthesis of secondary metabolites (Fig. 1D).

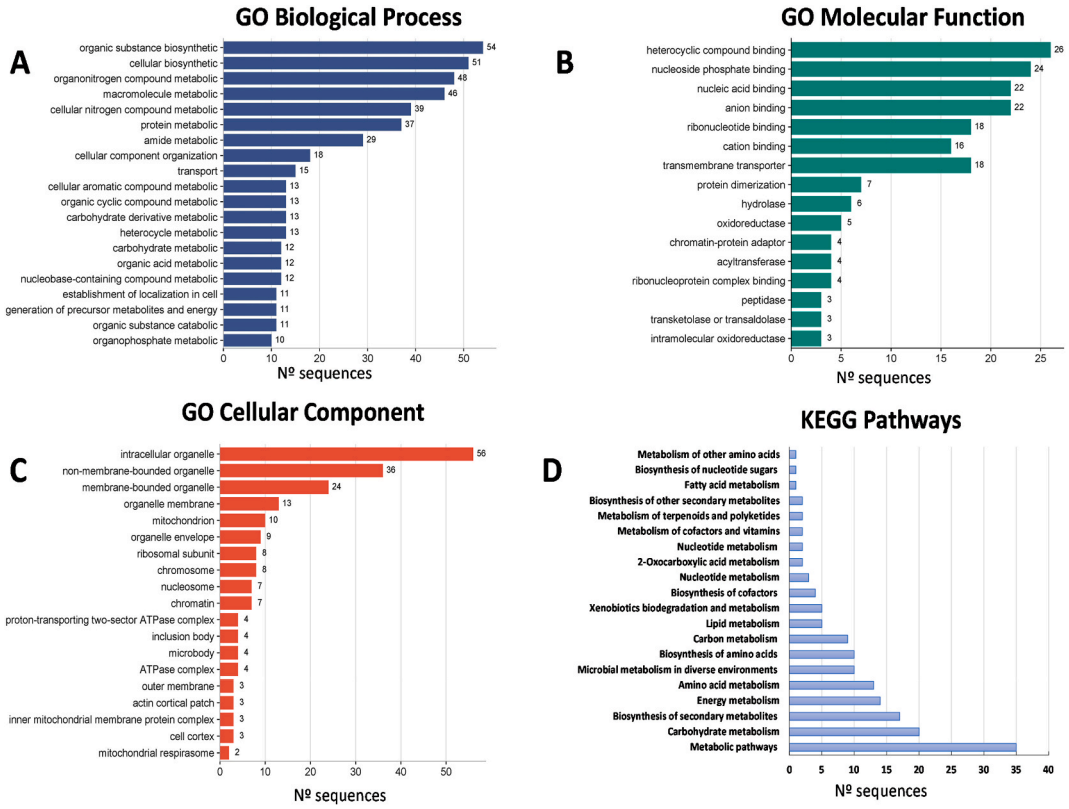


Fig. 1. GO functional classification and KEGG pathways of HSPeS cDNA library. GO categories are organized in decreasing order according to their number of sequences. The X axis shows the number of sequences. The Y axis denotes GO terms. All of the GO terms are grouped into three ontologies: (A) is for biological process, (B) is for molecular function and (C) is for cellular component. (D) KEGG pathway sorted in increasing order according to their number of sequences.

On the other hand, it is worth highlighting the large number of enzymes involved, represented mainly by oxidoreductases and hydrolases and transferases and to a lesser extent, lyases, isomerases, translocases and ligases that are only represented in 3% each. (Fig. 2).

3.3. Evaluation of gene expression profiling during orange discs interaction

Following sequence analysis of the HSPeS cDNA library, 24 genes were selected taking into account both their representation, determined by the number of reads, and their putative function based on their sequence homology with the genome of *P. expansum* Pex1 and of other *P.expansum* strains available in databases (Table 3).

To clarify the effectiveness of the HSPeS library, gene expression was performed at early stages (24h) during an incompatible interaction using samples of Pex1 interacting with orange discs (dPex1) and as a control Pex1 grown in PDB (Pex1). Balloon plot results showed that all genes evaluated, except for one coding an aldolase type TIM barrel (PEX1_014070), had a higher gene expression in *P. expansum* (Pex1) during their interaction with orange discs compared to *P. expansum* (Pex1) in axenic growth, demonstrating the efficiency of the subtraction (Fig. 3).

Induction analysis revealed that the highest induction corresponds to an ABC transporter (PEX1_089030), followed by 3-hydroxyacyl-CoA dehydrogenase (PEX1_083600), a glycoside hydrolase (PEX1_055640) and the MAGE protein (PEX1_067030). The rest of genes exhibited between 2- and 4-fold induction. Four of them were DEGs but rate of induction was very low (PEX1_034010, PEX1_049570, PEX1_016860 and PEX1_036150). Moreover, transcription abundance was different depending on the gene evaluated. The genes with less transcription rate correspond to PEX1_035540; PEX1_088460, PEX1_039540, PEX1_097120, PEX1_014410, PEX1_089030 while the highest correspond to a glycoside hydrolase (PEX1_056420) followed by a helicase (PEX1_018140) follow by an aldolase (PEX1_94410), a chaperone (PEX1_032120), an endoglucanase (PEX1_094750) and a peptidase (PEX1_ 034030) (Fig. 3, Fig. S1).

3.4. Gene transcription analysis during incompatible infection

A more in-depth study of the gene transcription profile was carried out for the 24 selected genes, analyzing their expression over time (1–3 days post inoculation) in axenic growth for two different *P. expansum* strains Pex1 and Pex2 (with high and medium grade of virulence, respectively) and during Pex1 incompatible infection of orange fruits (Figs. 4–5).

The results on heat map and balloon plot showed that in most genes, transcriptional abundance *in vitro* conditions increase in Pex1 strain compared to Pex2 with rising trend over time, with the exception of PEX1_01906, PEX1_ 035540, PEX1_036150 and PEX1_067030 in which expression rate was similar for both strains (Fig. 4).

Magnitude of expression level was different for each gene as we previously detected during evaluation in orange discs. Only sixteen of 24 selected genes showed increased expression during orange infection (Figs. 4–5). Of all of them, PEX1_55640, PEX1_051270, PEX1_016620, PEX1_014410 and PEX1_088780 should be noted for their early and sustained induction over time, except in the case of PEX1_088780, whose induction occurs exclusively at 24 h. A striking aspect is that the majority of those that presented induction during infection showed their highest transcriptional level at 3 dpi (late expression) except in the case of PEX1_016620, whereas induction decreased over time. Of all the genes studied and induced during incompatible infection, 4 of them PEX1_89030 (ABC transporter), PEX1_ 055640 (PG) PEX1_014410 (GST) and PEX1_88780 (ATPase subunit) showed almost exclusive expression during infection (Fig. 5).

Expression analysis comparison of different genes which assigned function was the same was carried out for 3 ATPases and 3 aldolases. As shown in Fig. 6, the expression pattern of each of the proteins within a given group was different. In the case of the ATPases, while PEX1_019060 did not show differences between both isolates of *P. expansum in vitro* nor did it show induction during infection, the other two PEX1_97120 and PEX1_088780 showed induction during infection with a very different transcriptional profile. PEX1_97120 showed its highest induction at 3 dpi while PEX1_088780 was exclusive during the first 24 h of infection. It should also be noted that the degree of transcription was very different, with the highest being PEX1_088780 and the lowest being PEX1_019060.

Likewise, the three genes that encode aldolases showed very different transcription profiles. One of them, PEX1_014070 was not induced during the infection, although it presented variances between both isolates, Pex1 and Pex2 during its growth *in vitro*. On the contrary, PEX1_094410 and PEX1_035540 were induced during infection in an increasing trend over time and presented opposite

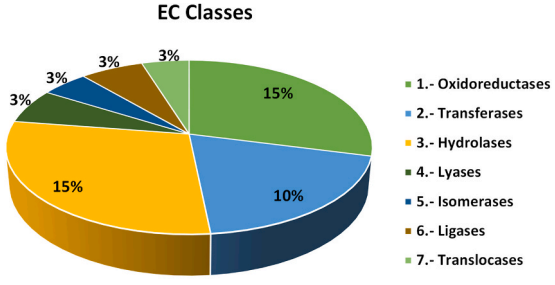


Fig. 2. Enzyme's categories represented in HSPeS library.

Table 3
List of selected genes of HSPeS cDNA subtracted library. Those that presented *in vivo* induction are highlighted in bold.

Gene	Description	NºReads	ID
XP_016599146.1	ATPase, AAA-type	31229	PEX1_019060
XP_016603435.1	Helicase	11678	PEX1_018140
XP_016593556.1	Aldolase-type TIM barrel	8283	PEX1_094410
XP_016592836.1	Molecular chaperone IbpA, HSP20 family	6457	PEX1_032120
XP_016593980.1	3-hydroxyacyl-CoA dehydrogenase	5531	PEX1_083600
XP_016595822.1	Barwin-related endoglucanase	5211	PEX1_094750
XP_016595155.1	Glycoside hydrolase, family 28	2309	PEX1_055640
XP_016602855.1	Fibrillarin	1557	PEX1_034010
XP_016602796.1	Histone-fold	1497	PEX1_049570
XP_016593359.1	Phospholipid/glycerol acyltransferase	482	PEX1_051270
XP_016603103.1	Aldolase-type TIM barrel	423	PEX1_035540
XP_016596720.1	Alcohol dehydrogenase superfamily, zinc-type	203	PEX1_016620
XP_016596933.1	Glyceraldehyde/Erythrose phosphate dehydrogenase family	191	PEX1_056420
XP_016593751.1	Aldolase-type TIM barrel	133	PEX1_014070
XP_016596744.1	Choline kinase	28	PEX1_016860
XP_016594353.1	Thiamine pyrophosphate enzyme	14	PEX1_088460
XP_016598404.1	Nucleic acid-binding, OB-fold	13	PEX1_039540
XP_016603666.1	ATPase, F0/V0 complex, subunit C	11	PEX1_097120
XP_016596212.1	Small GTPase superfamily, ARF/SAR type	9	PEX1_036150
XP_016596523.1	MAGE protein	6	PEX1_067030
XP_016602853.1	Peptidase M16, core	5	PEX1_034030
XP_016603413.1	CDR ABC transporter	4	PEX1_089030
XP_016602900.1	Glutathione S-transferase/chloride channel	2	PEX1_014410
XP_016601596.1	V-type ATPase subunit D	2	PEX1_088780

induction profiles between both *P. expansum* strains during axenic growth. As with ATPase genes, it should also be noted that the degree of transcription was very dissimilar, with the highest being PEX1_094410 and the lowest being PEX1_035540 (Fig. 6).

4. Discussion

P. expansum is the main pathogenic agent causing blue rot in pome fruits during postharvest [26]. In the last decade, studies on the molecular characteristics of *P. expansum* have made huge advance, focusing on the study of the pathogenicity of *P. expansum* and patulin biosynthesis in order to facilitate the expansion of new control strategies [8]. However, aspects related to the host specificity that determines its wide range of hosts and specifically identifying specific factors related to it, had not been addressed until now. Knowledge of the mechanisms of both host specificity and virulence constitutes a very important step to provide new alternative control treatments to the fungicides used.

Although *P. expansum* is not a pathogen of citrus fruits, is able to infect citrus fruit in some conditions [3,27]. Therefore, in this work through the incompatible interaction orange-*P. expansum*, genes associated in host recognition have been described using the suppressive subtractive hybridization (SSH) technique [17]. The characterization of the HSPeS subtracted cDNA library by means of massive sequencing allowed the identification of a total of 154 differentially expressed genes (DEGs) providing a wide transcriptional outlook. At 1 dpi, "substance biosynthetic, macromolecule and protein metabolic," were the most differentially expressed category showed in the GO sorting system. Organic substance biosynthetic was the most significantly enriched biological processes in the set of genes upregulated whereas heterocyclic compound was the most significant molecular function. A faster check of the genes upregulated during the orange interaction process highpoints a profusion of genes related to pathogenicity, such as ATPases, cell wall–degrading enzymes (CWDE) including hydrolases, and oxidoreductases. The highest expression values were found in PEX1_088780 encoding an ATPase showing exclusive expression at early stages (1 dpi), followed by PEX1_055640 and PEX1_016620 encoding a polygalacturonase and an alcohol dehydrogenase, respectively.

P. expansum most represented EST (PEX1_019060) coded an ATPase. Usually, ATPases can act acidifying an extensive collection of intracellular organelles and drives protons across the plasma membranes of many cell categories [28]. In *P. expansum*, numerous genes are regulated by environmental pH and one of the keys to the success of pathogens during their interaction with the host is precisely due to pH modulation [29,30]. In this work, eight different ATPases were identified and three of them were selected for the study of their transcriptional profile. All of them showed different expression profiles but two were involved in incompatible infection since gene expression was induced *in vivo*. Of this two, one had late expression (3dpi) while PEX1_088780 showed early expression and also the highest transcription rate, playing a relevant role during the pathogen-host interaction. Moreover, *P. expansum* ATPases could be negatively controlled by the transcription factor *PeSte12* which determines virulence and conidiation, both relevant aspects for fungal progression [13].

P. expansum colonizes the fruit through natural or produced lesions, before, during and after harvest [31]. Fungal pathogens have developed numerous virulence strategies to effectively infect host plants and one of them is to suppress the innate immune responses of the plant, thus modulating the physiology of the host in order to favor infection and the spread of the disease [32]. These approaches include a wide range of virulence factors also called "effectors", secreted as part of the initiation of host-microbe interactions that act as chemical or biochemical signals which play an important role in plant infection [33,34]. Secondary metabolites allow fungi to generate

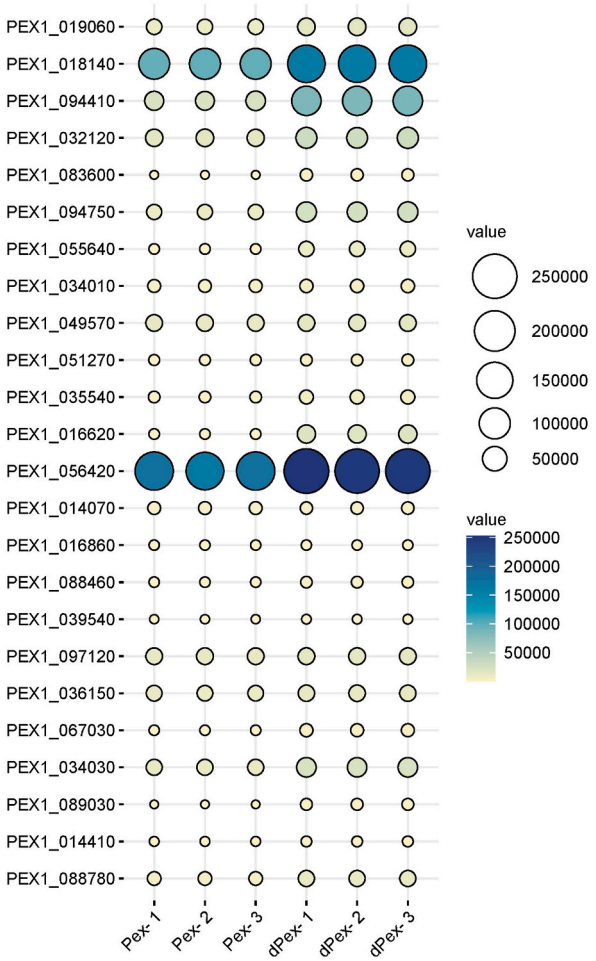


Fig. 3. Ballon plot of the 24 selected genes across all treatments sorted by RGE. Condition Pex (1–3) correspond to three biological replicas of *P. expansum* CECT 20906 grown for 24 h in PDB media on 24-well plate. Condition dPex (1–3) correspond to three biological replicas of *P. expansum* CECT 20906 grown for 24h interacting with orange discs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a wide diversity of chemical compounds that determine fungal infectivity, host specificity, and adaptation to ecological niches. CWDEs cause the softening and deterioration of fruits through the break of the cell wall and the degradation of secondary metabolites of the host, which is why they represent one of the most crucial pathogenic factors in postharvest pathogens [35]. In this work, PEX1_055640, which encodes a glycoside hydrolase (polygalacturonase) is one of the most notable genes both for its representation and also because its transcriptional rate and gene expression trend that increases over time exclusively during infection. Several RNA-seq studies carried out with apples infected with *P. expansum* support the relevance of CWDEs in fungal virulence since are associate to the most induced genes during infections [14,15]. The expression analysis data established the induction of two polygalacturonase-encoding genes through infection [36]. Furthermore, genes that encode a pectate lyase (PEX1_083670) and a polygalacturonase (PEX1_055640), which corresponded to pectinases not previously described, were overexpressed during apple infection [5]. Interestingly, among the effectors there is a large proportion that correspond to genes for enzymes that degrade the cell wall, which include lectins/glucanases similar to concanavalin A, pectin lyase and pectinesterase. A new effector gene, Peclg, has been identified and showed that it is relevant in conidiation, germination and environmental stress response [37]. Interestedly, in this study this group is also represented by an endoglucanase (PEX1_094750) and by another Glycoside hydrolase of family 12 that could have similarity with Concanavalin A-like lectins/glucanases (PEX1_081540). Our data supports that CWDEs perform an important protagonist in the early stages of the infection as it has been reported by Wang et al. [15] in which concanavalin A is involved in various biological processes, such as the growth and extension of the cell wall and in the suppression or prevention of plant immune responses [38].

Moreover, our results agree with those previously described in the infection of *P. expansum* at 1 dpi [5] highlighting exosome, mitochondrial biogenesis, ribosome, ubiquitin system and within the enzymes the same groups of hydrolases, transferases, oxidoreductases, ligases and isomerases. Gene expression in early stages of infection of *P. expansum* on apples allows uncover how the host-pathogen interaction occurs. *P. expansum* absorbs nutrients from the apple (elementary metabolism), adapts to the situation

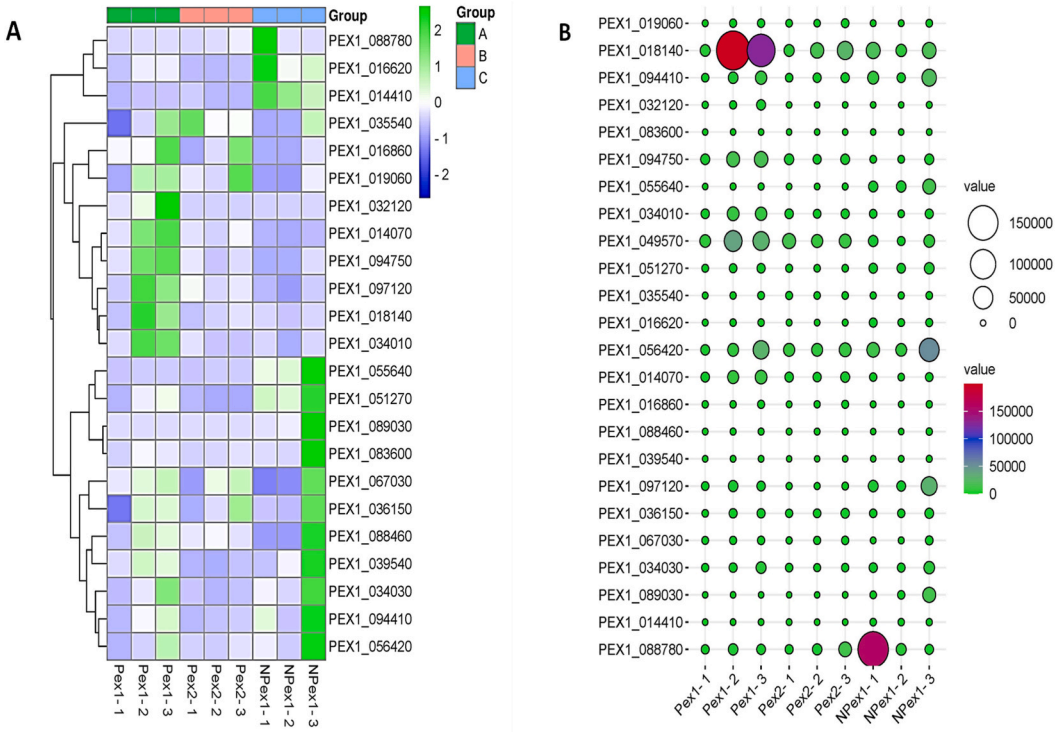


Fig. 4. **A**: Heat map showing 24 selected differentially expressed genes (DEGs) for experiments A B and C sorted by RGE. Hierarchical clustering is included for genes (y axis). Group A and B correspond to Pex1 and Pex2 respectively, grown in PDB media. Group C correspond to Pex1 infecting orange fruits. (1–3) correspond to 1-, 2- and 3-days assay. Colour scaling was applied to the genes (row Z-score represented). **B**: Ballon plot representing 24 selected genes across all treatments organized by RGE. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(stimulus answer), and prepares for wound penetration. Then, *P. expansum* progressively develops process of detoxification and reproduction [30]. Another aspect of interest is that the host, citrus fruits, in an incompatible interaction, releases a significant amount of H_2O_2 at the site of infection as defense mechanisms against *P. expansum* [39]. The presence of detoxifying genes such as two glutathione (GSH)-S-transferase and a thioredoxin identified in this work might be justified, specially, PEX1_014410 that revealed high induction during incompatible interaction. Besides, a potential link between oxidative stress response and virulence in *P. expansum* has been reported including several genes (glutathione (GSH)-S-transferase and thioredoxin) directly involved in the detoxification of ROS [40].

Effectors contribute to the virulence of *P. expansum* [6] and Levin et al. [41] showed that a subtilisin-related peptidase *Peprt* gene plays a role in *P. expansum* infection. In this work, two peptidases were identified in host specificity assays and one of them (PEX1_034030) showed increase gene expression during infection. It is worth noted that PEX1_096670 that act as an effector, which was found in this work during incompatible citrus infection, corresponds to a previously identified gene specifically expressed in the apple-*P. expansum* interaction [36] and in RNA-seq studies in the infection of *P. expansum* at 24h [5].

Another group with great relevance includes the aldolase-type TIM barrel family associated to different metabolic processes. Three different genes were analyzed and, as occurred in the ATPases, the expression profiles were very different. Although two of them exhibited higher induction during infection, only PEX1_094410 showed a high transcriptional rate. Some genes belonging to the same family showed that the gene expression pattern was determined by pH and environmental conditions [30]. Modulation of pH could be an effective strategy to assure the success of the pathogen during the host-pathogen interaction since optimal environmental situations (pH, nutrients) allow the efficient use of enzymes [30].

Our study reveals that many of the genes involved in the incompatible *P. expansum*-orange infection are similar to those found in the compatible *P. expansum*-apple infection, involving CWDEs that facilitate access to nutrients, genes such as ATPases and aldolases, modulated by pH that allow adaptation to environmental or stress conditions and above all, the “effectors” that are the essential component in colonization and subsequent infection. The achievement of colonization and infection lies in the ability of the pathogen to modify host plants to obtain the nutrients necessary for growth. All of these processes, unlike infection, occur in early stages, being the key to whether the infection progresses or not. Of all the genes analyzed whose expression was differential in the incompatible *P. expansum*-orange infection, only sixteen were upregulated during orange infection and seven seems to be related to host specificity since no correlation has been reported to fungal virulence during the compatible *P. expansum*-apple interaction. Most of them (PEX1_083600, PEX1_51270, PEX1_56420, PEX1_088460) agree with enzymes that mediate in the basic metabolism that, in stress

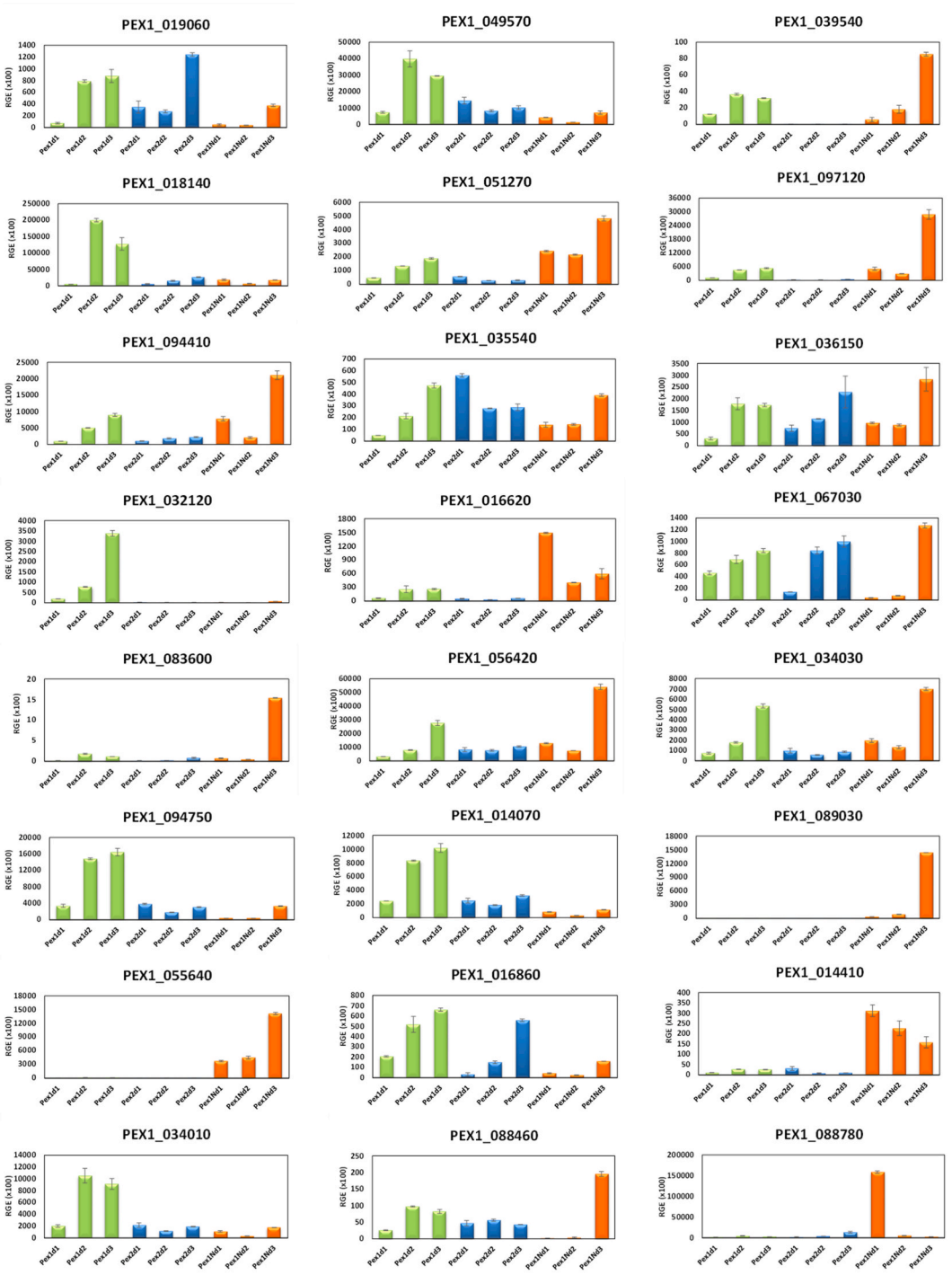


Fig. 5. Analysis of relative gene expression (RGE) of 24 selected genes. Temporary evaluation of gene transcription of Pex1, Pex2 strains grown in PDB liquid culture at 25 °C and Pex1N corresponds gene expression of Pex1 during orange infection. In all cases: d1, d2 and d3 agree with 1 dpi, 2 dpi and 3 dpi, respectively. β-tubulin was used reference gene. Error bars indicate standard deviations of three biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conditions, assurance the supply of nutrients. With this study we provide a broad perspective that reveals shared mechanisms of *P. expansum* during infection regardless of the host and specific mechanisms to adapt to each host that will explain its wide range of hosts.

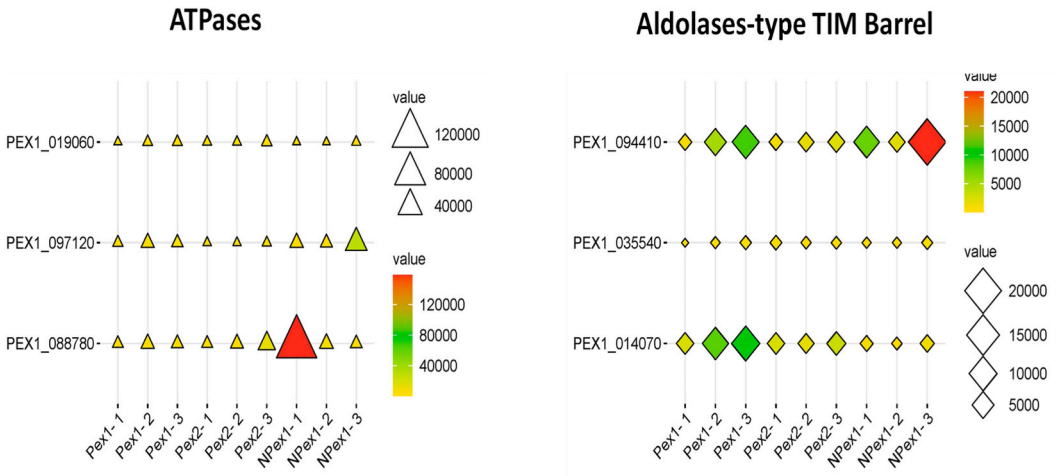


Fig. 6. Plots showing relative gene expression (RGE) of 3 different genes that code for the same putative function, ATPase and Aldolase. Time course assessment of gene expression of Pex1, Pex2 strains grown in PDB liquid culture at 25 °C and NPex1coresponds to time course of gene expression of Pex1 during orange infection. In all cases: 1, 2 and 3 correspond to 1 dpi, 2 dpi and 3 dpi, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusions

In summary, the use of an SSH approach in combination with a wide transcriptional outlook of differential genes of *P. expansum* during an incompatible infection showed genes with sustained induction over time. These genes encode enzymes related to the adaptation of the pathogen to the environment and the host to obtain the nutrients necessary for its growth and development and are associated with the basal metabolism of the pathogen under stress conditions and not related to the virulence of *P. expansum* under conditions of compatible interactions. Closer examination of the genes upregulated during infection in orange highlighted a large number of early expressed genes associated with virulence, such as ATPases, cell wall degrading enzymes (CWDEs), oxidoreductases and other shared effectors in the infection of pome fruits.

Data availability

The datasets generated during the current study listed in Table S2. The other data obtained during the current study will be available in Institutional Repository DIGITAL CSIC.

Funding

This research was funded by AGL2008-04828-C03 and AGL2011-30519-C03-02 projects from the Ministry of Science and Innovation (Spain). Marta de Ramón-Carbonll was recipient of INIA fellowship.

CRediT authorship contribution statement

Marta de Ramón-Carbonell: Investigation, Formal analysis. **Paloma Sánchez-Torres:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dr. Talens-Perales for his valuable help and advice on bioinformatic analysis.

We acknowledge the award of the Spanish government MCIN/AEI to the IATA-CSIC as Center of Excellence Accreditation Severo Ochoa (CEX2021-001189-S/MCIU/AEI/10.13039/501100011033).

We acknowledge the help of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29124.

References

[1] I. Julca, S. Droby, N. Sela, M. Marcet-Houben, T. Gabaldón, Contrasting genomic diversity in two closely related postharvest pathogens: *Penicillium digitatum* and *Penicillium expansum*, Genome Biol Evol 8 (1) (2016) 218–227.

[2] L. Gong, Y. Liu, Y. Xiong, T. Li, C. Yin, J. Zhao, J. Yu, Q. Yin, V.K. Gupta, Y. Jiang, X. Duan, New insights into the evolution of host specificity of three *Penicillium* species and the pathogenicity of *P. italicum* involving the infection of Valencia orange (*Citrus sinensis*), Virulence 11 (1) (2020) 748–768.

[3] L. Vilanova, I. Viñas, R. Torres, J. Usall, A.M. Jauset, N. Teixidó, Infection capacities in the orange-pathogen relationship: compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions, Food Microbiol. 29 (1) (2012) 56–66.

[4] R.R. Stange, S.L. Midland, J.J. Sims, T.G. McCollum, Differential effects of citrus peel extracts on growth of *Penicillium digitatum, P. italicum,* and *P. expansum*, Physiol. Mol. Plant Pathol. 61 (2002) 303–311.

[5] A.R. Ballester, M. Marcet-Houben, E. Levin, N. Sela, C. Selma-Lázaro, L. Carmona, M. Wisniewski, S. Droby, L. González-Candelas, T. Gabaldón, Genome, Transcriptome, and Functional Analyses of *Penicillium expansum* provide new insights into secondary metabolism and pathogenicity, Mol. Plant Microbe Interact. 28 (2015) 232–248.

[6] D. Luciano-Rosario, N.P. Keller, W.M. Jurick, *Penicillium expansum*: biology, omics, and management tools for a global. Postharvest pathogen causing blue Mould of pome fruit, Mol. Plant Pathol. 21 (2020) 1391–1404.

[7] B. Li, Y. Zong, Z. Du, et al., Genomic characterization reveals insights into patulin biosynthesis and pathogenicity in *Penicillium* species, Mol. Plant Microbe Interact. 28 (6) (2015) 635–647.

[8] B.Q. Li, Y. Chen, Z.Q. Zhang, G.Z. Qin, T. Chen, S.P. Tian, Molecular basis and regulation of pathogenicity and patulin biosynthesis in *Penicillium expansum*, Compr. Rev. Food Sci. Food Saf. 19 (2020) 3416–3438.

[9] D. Luciano-Rosario, H. Peng, V.L. Gaskins, J.M. Fonseca, N.P. Keller, W.M. Jurick, Mining the *Penicillium expansum* genome for virulence genes: a functional-based approach to discover novel Loci mediating blue mold decay of apple fruit, J. Fungi 9 (2023) 1066.

[10] Y. Chen, B. Li, X. Xu, Z. Zhang, S. Tian, The pH-responsive PacC transcription factor plays pivotal roles in virulence and patulin biosynthesis in *Penicillium expansum*, Environ. Microbiol. 20 (2018) 4063–4078.

[11] C. El Hajj Assaf, S.P. Snini, S. Tadrist, S. Bailly, C. Naylies, I.P. Oswald, S. Lorber, O. Puel, Impact of veA on the development, aggressiveness, dissemination and secondary metabolism of *Penicillium expansum*, Mol. Plant Pathol. 19 (2018) 1971–1983.

[12] D. Kumar, S. Barad, Y. Chen, X. Luo, J. Tannous, A. Dubey, N. Glam Matana, S. Tian, B. Li, N. Keller, et al., LaeA regulation of secondary metabolism modulates virulence in *Penicillium expansum* and is mediated by sucrose, Mol. Plant Pathol. 18 (2017) 1150–1163.

[13] P. Sánchez-Torres, L. Vilanova, A.R. Ballester, M. López-Pérez, N. Teixidó, I. Viñas, J. Usall, L. González-Candelas, R. Torres, Unravelling the contribution of the *Penicillium expansum* PeSte12 transcription factor to virulence during apple fruit infection, Food Microbiol. 69 (2018) 123–135.

[14] J. Tannous, D. Kumar, N. Sela, E. Sionov, D. Prusky, N.P. Keller, Fungal attack and host defence pathways unveiled in near-avirulent interactions of *Penicillium expansum* creA mutants on apples, Mol. Plant Pathol. 19 (2018) 2635–2650.

[15] K. Wang, X. Zheng, X. Zhang, L. Zhao, Q. Yang, N.A.S. Boateng, J. Ahima, J. Liu, H. Zhang, Comparative transcriptomic analysis of the interaction between *Penicillium expansum* and apple fruit (*Malus pumila* Mill.) during early stages of infection, Microorganisms 7 (2019) 495.

[16] M. de Ramón-Carbonell, P. Sánchez-Torres, Involvement of *Penicillium digitatum PdSUT1* in fungicide sensitivity and virulence during citrus fruit infection, Microbiol. Res. 203 (2017) 57–67.

[17] M. López-Pérez, A.R. Ballester, L. González-Candelas, Identification and functional analysis of *Penicillium digitatum* genes putatively involved in virulence towards citrus fruit, Mol. Plant Pathol. 16 (2015) 262–275.

[18] L. Diatchenko, Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, P.D. Siebert, Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, Proc. Natl. Acad. Sci. USA 93 (1996) 6025–6030.

[19] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[20] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows–Wheeler transform, Bioinformatics 25 (2009) 1754–1760.

[21] H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, 1000 genome project data processing Subgroup, the sequence alignment/map format and SAM tools, Bioinformatics 25 (2009) 2078–2079.

[22] D.J. Winter, rentrez: an R package for the NCBI eUtils API, The R Journal 9 (2017) 520–526.

[23] R.P. Huntley, T. Sawford, P. Mutowo-Muellenet, A. Shypitsyna, C. Bonilla, M.J. Martin, C. O'Donovan, The Goa database: gene Ontology annotation updates for 2015, Nucleic Acids Res. 43 (2014) 1057–1063.

[24] M. de Ramón-Carbonell, M. López-Pérez, L. González-Candelas, P. Sánchez-Torres, MFS1 transporter contributes to *Penicillium digitatum* fungicide resistance and fungal virulence during citrus fruit infection, J. Fungi 5 (2019) 100.

[25] M. de Ramón-Carbonell, P. Sánchez-Torres, The transcription factor *PdSte12* contributes to *Penicillium digitatum* virulence during citrus fruit infection, Postharvest Biol. Technol. 125 (2017) 129–139.

[26] L. Zhong, J. Carere, Z. Lu, F. Lu, T. Zhou, Patulin in apples and apple-based food products: the burdens and the mitigation strategies, Toxins 10 (2018) 475.

[27] J.P. Louw, L. Korsten, Pathogenicity and host susceptibility of Penicillium spp. on citrus, Plant Dis. 99 (2015) 21–30.

[28] S.H. Lee, A.P. Singh, G.C. Chung, S.J. Ahn, E.K. Noh, E. Steudle, Exposure of roots of cucumber (*Cucumis sativus*) to low temperature severely reduces root pressure, hydraulic conductivity and active transport of nutrients, Physiol. Plantarum 120 (2004) 413–420.

[29] S. Barad, E.A. Espeso, A. Sherman, D. Prusky, Ammonia activates pacC and patulin accumulation in an acidic environment during apple colonization by *Penicillium expansum*, Mol. Plant Pathol. 17 (2016) 727–740.

[30] S. Barad, N. Sela, D. Verma, A. Dubey, G. Glam, A. Sherman, D. Prusky, Fungal and host transcriptome analysis of pH-regulated genes during colonization of apple fruits by *Penicillium expansum*, BMC Genom. 17 (2016) 330.

[31] M. Wenneker, B.P.H.J. Thomma, Latent postharvest pathogens of pome fruit and their management: from single measures to a systems intervention approach, Eur. J. Plant Pathol. 156 (2020) 663–681.

[32] Y. Wang, R.N. Pruitt, T. Nurnberger, Y. Wang, Evasion of plant immunity by microbial pathogens, Nat. Rev. Microbiol. 20 (2022) 449–464.

[33] C. Selin, T.R. de Kievit, M.F. Belmonte, W.G.D. Fernando, Elucidating the role of effectors in plant-fungal interactions: progress and challenges, Front. Microbiol. 7 (2016) 600.

[34] J.G. de Moraes Pontes, L.S. Fernandes, R.V. Santos, L. Tasic, T.P. Fill, Virulence factors in the phytopathogen-host interactions: an overview, J. Agric. Food Chem. 68 (2020) 7555–7570.

[35] C.P. Kubicek, T.L. Starr, N.L. Glass, Plant cell wall–degrading enzymes and their secretion in plant-pathogenic fungi, Annu. Rev. Phytopathol. 52 (2014) 427–451.

[36] P. Sánchez-Torres, L. González-Candelas, Isolation and characterization of genes differentially expressed during the interaction between apple fruit and *Penicillium expansum*, Mol. Plant Pathol. 4 (2003) 447–457.

[37] J. Zhou, W. Gong, T. Tu, J. Zhang, X. Xia, L. Zhao, X. Zhou, Y. Wang, Transcriptome analysis and functional characterization reveal that *Peclg* gene contributes to the virulence of *Penicillium expansum* on apple fruits, Foods 12 (3) (2023) 479.
[38] E.L. Bradley, B. Okmen, G. Doehlemann, B. Henrissat, R.E. Bradshaw, C.H. Mesarich, Secreted glycoside hydrolase proteins as effectors and invasion patterns of plant-associated fungi and oomycetes, Front. Plant Sci. 13 (2022) 853106.
[39] D. Macarisin, L. Cohen, A. Eick, G. Rafael, E. Belausov, M. Wisniewski, S. Droby, *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit, Phytopathology 97 (2007) 1491–1500.
[40] Y. Chen, Y. Zhang, D. Xu, Z. Zhang, B. Li, S. Tian, PeAP1-mediated oxidative stress response plays an important role in the growth and pathogenicity of *Penicillium expansum*, Microbiol. Spectr. 11 (5) (2023) e0380822.
[41] E. Levin, A. Kishore, A.R. Ballester, G. Raphael, O. Feigenberg, Y. Liu, J. Norelli, L. Gonzalez-Candelas, M. Wisniewski, S. Droby, Identification of pathogenicity-related genes and the role of a subtilisin-related peptidase S8 (PePRT) in authophagy and virulence of Penicillium expansum on apples, Postharvest Biol. Technol. 149 (2019) 209–220.